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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No

22091-701

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First Named Inventor or Application Identifier

Schenk, John L.

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APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents:

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1. ☒ Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)
2. ☒ Specification (preferred arrangement set forth below) [Total Pages 60]
- Descriptive title of the Invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☐ Drawings (35 USC 113) [Total Sheets]
4. ☐ Oath or Declaration [Total Pages 3]
- a. ☒ New
- b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
(Note Box 5 below)
1. ☐ **DELETION OF INVENTOR(S)**
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)
5. ☐ Incorporated By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein
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8. ☒ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement (when there is an assignee) ☒ Power of Attorney (in Declaration)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
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Docket No. 22091-701

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John L. Schenk
Application No.: Unknown
Filed: December 17, 1999
For: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 C.F.R. §§ 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN**

I hereby declare that I am

- ☐ () the owner of the small business concern identified below:
☒ (X) an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: XY, Inc.
ADDRESS: 3801 W. Rampart Road
ARBL Building, CSU Foothills Research Campus
Fort Collins, CO 80523

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 C.F.R. § 121.3-18, for purposes of paying reduced fees under § 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled METHOD OF CRYOPRESERVING SELECTED SPERM CELLS by inventor, John L. Schenk

described in

- (X) the specification filed herewith
() application Serial No. _____, filed _____.
() Patent No. [_____], issued [Date].

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

Full Name: _____

Address: _____

() Individual () Small Business Concern () Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 C.F.R. § 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of person signing: DR. MERVYN JACOBSON

Title of person
other than owner: PRESIDENT & CEO.

Address of person signing: XY, Inc.
3801 W. Rampart Road
ARBL Building, CSU Foothills Research Campus
Fort Collins, CO 80523

Signature: [Signature]

Date: DEC 23rd 1999.

¹NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27)

METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 The applications claims the benefit of U.S. Provisional Application No. 60/167,423, filed November 24, 1999.

FIELD OF THE INVENTION

The invention relates to a method for freezing sperm selected for a particular characteristic, as well as to a frozen selected sperm sample and methods of using such a sample.

- 10 The invention is particularly useful for preserving sex-selected sperm.

BACKGROUND OF THE INVENTION

- Over half a century ago, artificial insemination was introduced in the United States as a commercial breeding tool for a variety of mammalian species. Although artificial insemination was initially limited to regions relatively close to the site of sperm collection, advances in the cryopreservation and storage of sperm have facilitated widespread distribution and commercialization of sperm intended for artificial insemination or in vitro fertilization.

- Further improvements in mammalian sperm collection, selection, cryopreservation, storage, and handling techniques have enhanced the ability of breeders to produce animals having desired traits. For example, advances in selection of mammalian sperm based on slight differences in physical characteristics has made it possible to separate sperm based on sex-type, that is, to select for cells containing either the X or Y chromosome. This technique allows the breeder to manipulate the relative percentage of X- or Y-type sperm in a sample and thereby determine offspring sex. The ability to select sperm based on sex-type or any other desirable characteristic provides an important tool for accelerating genetic progress, increasing production efficiency, and achieving greater flexibility in livestock management. Full exploitation of this tool, however, depends on the ability to freeze and store selected sperm.

- A variety of methods are available for selecting cells; however, the selection and subsequent processing of sperm presents unique challenges because sperm are incapable of DNA repair and because of sperm morphology. Each sperm has an acrosome overlying the head and a tail, which are important for fertility and which are relatively susceptible to physical injury. In

addition, sperm fertility decreases with increasing time between collection and use. As most of the available selection methods involve physical stresses and take time, selected sperm are typically somewhat compromised compared to non-selected cells. Fertility may be further reduced if the selection technique involves significant dilution. It has been suggested that this “dilution effect” may be due to the loss of protective components in seminal plasma.

Flow cytometry is a particularly efficient selection method that has been employed for sorting sperm by sex-type. However, sorted sperm are subject to stresses beyond those normally encountered in standard artificial insemination or in vitro fertilization protocols. In particular, flow cytometry is time consuming, and, because of the physical constraints of flow cytometers, sperm must be diluted for sorting to levels that are not optimal for storage (usually to on the order of 10^5 - 10^6 /ml). Furthermore, sorted sperm intended for artificial insemination must be concentrated so that conventional packaging and delivery equipment can be used. The need for a concentration step thus exposes already somewhat compromised sperm to additional physical stresses.

The freezing of sperm also invariably reduces fertility, motility, and/or viability, and, although techniques for freezing unselected sperm are well known, no technique for cryopreservation of selected sperm has been described.

SUMMARY OF THE INVENTION

The present invention provides a method of cryopreserving sperm that have been selected for a specific characteristic. The method is particularly useful for cryopreserving sperm selected by a method that results in dilution of the sperm, since the method provides for the isolation of sperm from a selected sperm sample, followed by addition of a final extender to the isolated sperm to produce a suspension having a desired concentration of sperm. In a preferred embodiment, the method is employed to freeze sex-selected sperm. Although the cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred.

The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or

bison sperm. Also within the scope of the invention is a container including a frozen sperm sample according to the invention.

The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

DETAILED DESCRIPTION OF THE INVENTION

The present invention allows cryopreservation of sperm that have been selected for a particular characteristic, facilitating storage and/or shipment of selected sperm samples to sites distant from the collection site. Thawing yields viable sperm that can be used in procedures such as artificial insemination ("AI") and in vitro fertilization ("IVF"). This result was surprising because of the well-documented fragility of sperm. Prior researchers had demonstrated that the stresses associated with various selection methods or with cryopreservation resulted in significant losses in fertility and/or viability. The present inventors have demonstrated, for the first time, that pregnancies can be achieved with sperm that have been selected and then frozen.

The invention represents an important advance in livestock management, where selection of sperm for use in such procedures can be used to increase the production of offspring having desirable traits. For example, selection to obtain sperm carrying either the X or the Y chromosome allows control over offspring sex, which is advantageous for producers of animals such as dairy or beef cattle. Sex selection also finds application in breeding valuable (e.g., show or race horses) or endangered animals. The ability to freeze selected sperm, which the invention provides, will enable widespread use of such selection methods to, e.g., increase livestock production efficiency as well as quality.

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Definitions

The term "acrosome" or "acrosomal cap" refers to the cap that covers the anterior half of the head of sperm and that contains enzymes necessary for ovum penetration.

The term "sex-type" refers to the type of sex chromosome present in the sperm (i.e., the X or Y chromosome).

30

The term “capacitation” refers to the specific changes a sperm undergoes to develop the capacity to fertilize ova, such as enzymic changes on the surface of the acrosome that lead to release of acrosomal enzymes that facilitate penetration of the sperm into the ovum.

As used with reference to sperm, the term “cryoprotectant” refers to a molecule that
5 protects sperm during a freeze-thaw cycle, promoting survival and retention of fertilizing capacity.

The term “dilution effect” refers to the rapid decline in motility and/or viability of sperm when highly diluted.

As used herein, the term “selection” refers to a method whereby a sample is subdivided
10 based on presence or absence of a specific characteristic (unless context dictates otherwise). Thus, a “selected sperm sample” is a sample obtained by subjecting a source sample to selection for the specific characteristic. A selected sperm sample is therefore enriched, relative to the source sample, in sperm having the specific characteristic.

The term “sorting” is used herein to describe a selection method carried out using a
15 fluorescence-activated cell sorter (FACS).

The term “extender” refers to any medium that tends to preserve sperm viability. The term “extension” refers to the dilution of sperm with extender.

The term “initial extender” refers to a medium used to extend sperm prior to the isolation step of the method of this invention.

20 The term “final extender” refers to a medium used to extend sperm prior to the freezing step of the method of this invention.

An “organic substance” in an extender described herein is any organic substance that tends to reduce cold shock and preserve fertility of sperm.

An “energy source” in an extender described herein is any substance or substrate that
25 sperm can utilize for cell maintenance and/or motility.

The term “osmolality,” as used herein, is a measure of the osmotic pressure of dissolved solute particles in a an aqueous solution (e.g., an extender). The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of water.

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Cryopreservation Method

The invention provides a method of cryopreserving selected sperm includes the following steps:

- (1) obtaining a selected sperm sample;
- 5 (2) cooling the selected sperm sample;
- (3) isolating sperm from the selected sperm sample;
- (4) adding final extender to the isolated sperm to produce a suspension of sperm; and
- (5) freezing the suspension of sperm.

10 *Obtaining a Selected Sperm Sample*

The first step in the cryopreservation method of the invention encompasses obtaining a previously selected sperm sample, as well as subjecting a source sample to any suitable selection method. Sperm from any species can be selected and frozen according to the method of the invention. The method can be carried out with sperm from domesticated animals, especially
15 livestock, as well as with sperm from wild animals (e.g., endangered species). Preferably, the selected sperm sample contains mammalian sperm. Human sperm, bovine, equine, porcine, ovine, elk, and bison sperm are particularly preferred.

Generally, the selected sperm sample contains normal, viable sperm. To this end, the ejaculate from which the sperm are obtained typically has at least about 50%, and preferably at
20 least about 75% morphologically normal sperm. In these embodiments, generally at least about 40%, and preferably at least about 60% of the sperm in the ejaculate exhibit progressive motility.

A wide variety of methods for selecting cells from a mixed populations are available, including, for example, selection based on binding of cells or cell components with antibodies, antibody fragments, or other binding partners and differential staining.

25 The invention is exemplified herein with selection based on sex-type, and sex-selected sperm for use in the invention can be obtained using any selection strategy that takes advantage of slight differences in characteristics between X- and Y-type sperm. Exemplary sex-selection methods include magnetic techniques (see, e.g., U.S. Patent No. 4,276,139), columnar techniques (see, e.g., U.S. Patent No. 5,514,537) gravimetric techniques (see, e.g., U.S. Patent
30 No. 3,894,529, reissue Patent No. 32350, U.S. Patent Nos. 4,092,229, 4,067,965, and 4,155,831). Sex-selection based on differences in electrical properties is disclosed in U.S. Patent

No. 4,083,957, and techniques that select based on differences in electrical and gravimetric properties are discussed in U.S. Patent Nos. 4,225,405, 4,698,142, and 4,749,458. U.S. Patent Nos. 4,009,260 and 4,339,434 describe selection based on differences in motility. Biochemical techniques relying on antibodies are disclosed in U.S. Patent Nos. 4,511,661, 4,999,283, 3,687,803, 4,191,749, 4,448,767, whereas U.S. Patent Nos. 5,021,244, 5,346,990, 5,439,362, and 5,660,997 describe selection based on differences in membrane proteins.

Flow cytometry is a preferred method for separating cells from mixed populations based on differential staining with fluorescent dyes or binding to fluorescently labeled molecules, such as antibodies or nucleic acids. In fluorescence activated cell sorting ("FACS"), cells are "sorted" into different populations based on the fluorescence intensity upon irradiation. FACS can be used for sex-selection of sperm because the X chromosome contains slightly more DNA than the Y chromosome. When sperm are stained with a fluorescent DNA-binding dye, X-chromosome bearing sperm absorb more dye than Y-chromosome bearing sperm and the two populations can therefore can be separated by FACS. This strategy was discussed in U.S. Patent No. 4,362,246 and significantly expanded upon in U.S. Patent No. 5,135,759 (issued to Johnson). Separation has been enhanced through the use of high-speed flow cytometers, such as the MoFlo® flow cytometer produced by Cytomation, Inc. (Ft. Collins, CO) and described in U.S. Patent Nos. 5,150,313, 5,602,039, 5,602,349, and 5,643,796, as well as in PCT Publication No. WO 96/12171.

The selection method used to obtain the selected sperm sample is preferably one that preserves sperm viability. Because of the relative fragility of sperm, normal flow cytometry techniques should generally be modified for sorting sperm. More specifically, the flow cytometry entails staining, dilution, and interrogation of cells with light. All of these steps represent stresses that can reduce sperm viability. The sensitivity of sperm to these stresses can vary between species and even between individuals within species. Such sensitivities have either been documented or can readily be determined by empirical studies, such as those described in Examples 1-5.

Modifications that enhance viability are described the patent publications discussed above. For instance, procedures that provide improved sheath and collector systems for sorting sperm are disclosed in PCT Publication No. WO 99/33956 (Application No. PCT/US98/27909). Further, Examples 1-7 below describe exemplary procedures for staining and sorting sperm.

Example 3 describes a study of the effects of laser intensity and dye concentration of post-thaw motility of sorted frozen sperm. This study indicates that the use of lower laser intensities during sorting can increase post-thaw motility.

5 The selected sperm sample can contain a variety of components besides sperm and will often contain components added to protect the sperm during the selection process. In the case of FACS, the selected sperm sample can contain component(s) of the solutions used for staining and sorting (e.g., the sheath fluid and the catch buffer).

10 In addition, the selected sperm sample typically contains an extender or extender fraction. For example, “two-step” extenders including an “A fraction” lacking glycerol and a “B fraction” containing glycerol are well known. The A fraction is added to sperm first, followed by addition of an equal volume of the B fraction. For this step, the B fraction is often divided into at least two aliquots and added sequentially; e.g., the second B fraction aliquot is added 15 minutes after the first.

15 If no extender components are present, an extender or extender fraction is typically added to the selected sperm sample before the sperm are isolated from the sample. If only some extender components are present, additional components can optionally be added so that selected sperm sample includes a complete extender or an extender fraction before the isolation step. In exemplary embodiments, bovine sperm are flow-sorted so as to produce a selected sperm sample including the A fraction of an extender (see Examples 2, 3, and 4). If desired, the B fraction can
20 then be added to the selected sperm sample before the isolation step (see Example 5). The pre-isolation step extender (or extender fraction) is termed “the initial extender” to distinguish it from the “final extender” employed for the extension of isolated sperm before freezing. If the selected sperm sample was selected using FACS, the initial extender can be matched to the sheath fluid employed for sorting. Exemplary matched sheath fluids and extenders are described
25 in detail in Example 4.

An extender suitable for use in the selected sperm sample includes a physiologically acceptable carrier. The physiologically acceptable carrier is typically aqueous, and, in preferred embodiments, includes deionized water. Suitable extenders commonly comprise one or more of the following additional components: a cryoprotectant, a component that maintains osmolality
30 and buffers pH, an organic substance that prevents cold shock and preserves fertility of sperm, a detergent that acts synergistically with certain organic substances to enhance preservation of

sperm, an energy source that can be readily utilized by sperm, an antioxidant, which protects sperm from cold shock, a substance that facilitates sperm capacitation, and one or more antibiotics.

Although cryoprotectants useful in the invention are not limited to those acting by a particular mechanism, most conventional cryoprotectants act, at least in part, by reducing intracellular dehydration. Specifically, freezing is accompanied by an increase in solute concentration in the medium surrounding sperm. This increase in solute concentration draws water out of the cells, which increases intracellular electrolyte concentration. Exemplary cryoprotectants include glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and the like. The cryoprotectant suitable for use in a given extender can vary, depending on the species from which sperm are derived. For example, glycerol is suitable for use in cryopreservation of human and bovine sperm, but is generally not used for cryopreservation of porcine or rabbit sperm. Such preferences are well known for many commercially valuable sperm and can readily be determined empirically for other types of sperm.

The extender useful in the invention optionally includes one or more components that help maintain osmolality and provide buffering capacity. In preferred embodiments of the invention, the osmolality of the extender approximates that of physiological fluids. More preferably, the osmolality of the extender is in the range of about 280 mOsm to about 320 mOsm. The pH is also preferably within a physiologically acceptable range, more preferably in the range of about 6.5 to about 7.5.

Substances helpful in maintaining osmolality and pH within these ranges are well known in the art and can be added to the extender as a solid or already in solution. A buffer containing a salt, a carbohydrate, or a combination thereof can be employed for this purpose. Specific examples include sodium citrate, Tris[hydroxymethyl]aminomethane, and TES (N-Tris [Hydroxymethyl]methyl-2-aminoethanesulfonic acid), and monosodium glutamate buffers; milk; HEPES-buffered medium; and any combination thereof. The component employed to help maintain osmolality and provide buffering capacity in a particular application can vary depending on the other components of the extender and, in some cases, on the species from which the sperm are derived. The selection of such a component for use in the present invention is, however, within the level of skill in the art.

One or more organic substances that protect sperm against cold shock and help preserve fertilizing capacity can also be included in the extender. Such substances are well known and are sometimes described as “nonpenetrating cryoprotectants.” One skilled in the art can readily determine an organic substance suitable for a particular application of the cryopreservation method described herein. For example, organic substances containing protective constituents (e.g., lipoproteins, phospholipids, lecithin) that are believed to reduce the impact of cold shock and the dilution effect can be included in the extender. Suitable organic substances include disaccharides, trisaccharides, and any combination thereof. Exemplary organic substances include egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, cholesterol, and any combination thereof.

The extender can also include a detergent. Alkyl ionic detergents, such as sodium dodecyl sulfate (SDS), have been reported to act synergistically with egg yolk to enhance protection against cold shock. Other detergents useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular detergent for a specific application is within the level of skill in the art in light of the guidance provided herein. See, e.g., Example 5.

Preferably, the extender includes an energy source that is readily utilized by sperm. In the absence of an energy source, sperm may oxidize intracellular phospholipids and other cellular components. Thus, the inclusion of an energy source in the extender protects intracellular reserves and cellular components. As is well known in the art, sugars, particularly monosaccharides, provide a convenient energy source, although any conventional energy source can be employed in the extender. Exemplary monosaccharides useful in the extender include glucose, fructose, and/or mannose.

One or more antioxidants can optionally be included in the extender to provide additional protection against cold shock. Exemplary antioxidants include butylated hydroxytoluene (BHT), its derivatives, and the like. However, other antioxidants useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular antioxidant for a specific application is within the level of skill in the art in light of the guidance provided herein.

The extender can also contain a substance that facilitates sperm capacitation. A variety of capacitation facilitators are known in the art and any can be employed in the extender. Examples

include enzymes such as alpha amylase, beta amylase, beta glucuronidase, which can be used in combination, if desired.

Finally, the extender preferably includes an antibiotic, since substantial bacterial growth can threaten sperm viability and increase the risk of infection of the host in artificial insemination or in vitro fertilization procedures. Any of a variety of antibiotics useful in the cryopreservation of cells can also be employed in the extender. The selection of a suitable antibiotic depends on the species from which the sperm was obtained, the procedures involved in obtaining and handling the sperm sample, and the specific microorganism(s) to be targeted. Exemplary antibiotics include tylosin, gentamicin, lincomycin, spectinomycin, linco-spectin (a combination of lincomycin and spectinomycin), penicillin, streptomycin, and ticarcillin, which can be used alone or in combination. However, one skilled in the art can readily determine other antibiotics suitable for use in the extender.

Exemplary extenders are discussed in greater detail below and in the examples.

The sperm concentration is typically lower in the selected sperm sample than in the source sample, and, as indicated above, when FACS is employed, the dilution is significant. A typical sort based on sex-type can produce a sample containing sperm at 6×10^5 cells/ml catch buffer. As such a low concentration is not optimal for storage (at least for most species tested), the cryopreservation method of the invention generally concentrates the selected sperm sample.

Cooling the Selected Sperm Sample

The second step in the cryopreservation method entails cooling the selected sperm sample, typically, by reducing the temperature at a controlled rate. Cooling too rapidly can cause cold shock, which can result in a loss of membrane integrity and cell function. The severity of the effects of cold shock vary from species to species and depend on factors such as the rate of cooling and the temperature range. Under suitable controlled cooling conditions, the sperm are able to adapt to thermal effects. Example 2, among others, describes conditions for cooling bovine sperm, and determining suitable conditions for cooling sperm of other species is within the level of skill in the art.

In a preferred embodiment of the invention, the selected sperm sample is cooled typically from about 22°Celsius, to about 5°Celsius, and cooling is generally carried out over a period of about 60 minutes to about 24 hours, preferably over a period of about 90 minutes to about 240

minutes, and most preferably over a period of about 90 minutes to about 120 minutes. Cooling can be accomplished by any convenient method, including simply placing the selected sperm sample in a 5°Celsius environment.

5 *Isolation of Sperm Cells from the Selected Sperm Sample*

After initial extension of the selected sperm sample, sperm are isolated from the sample using any sufficiently gentle isolation method that provides at least about 50% recovery of sperm, more preferably about 75% to about 90% recovery of sperm, and most preferably about 80% to about 90% recovery of sperm. During the isolation step, the cooled sperm should
10 generally be kept cold, i.e., between about 1 and about 8°Celsius, and preferably close to 4 or 5°Celsius.

Any of a variety of methods suitable for recovering cells from a suspension can be used to isolate the sperm, including for example, filtration, sedimentation, and centrifugation. In an exemplary, preferred embodiment, the selected sperm sample is aliquoted into 50 ml tubes at
15 volumes not exceeding about 27 ml, and preferably between about 20 to about 27 ml. Centrifugation is carried out at about 4°Celsius, at about 850 x g, for about 20 minutes. Preferably, the centrifugation step provides at least about 50% to about 90% recovery of sperm, more preferably about 60% to about 90% recovery of sperm, and most preferably about 70% to about 90% recovery of sperm. After isolation, the supernatant is removed and the pellet is
20 suspended by vortexing gently or repeated aspiration at 4°Celsius. The sperm concentration is then typically determined (e.g., using a hemacytometer).

Final Extension of Isolated Sperm Cells

After isolation, the sperm are pooled, if desired, and extended with final extender to an
25 appropriate concentration for freezing. The concentration of sperm after the final extension and prior to freezing is preferably in the range of about 1×10^6 /ml to about 300×10^6 /ml, more preferably about 10×10^6 /ml to about 50×10^6 /ml, and most preferably about 10×10^6 /ml to about 20×10^6 /ml.

The description of the initial extender above also applies to the final extender, which can
30 be the same as or different from the initial extender. In particular embodiments, the composition

of the sperm sample extended with the final extender is substantially similar to (if not the same as) the composition of the sperm sample after addition of the initial extender.

In a preferred embodiment of the invention, an egg yolk-Tris extender is used. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); citric acid
5 and Tris[hydroxymethyl]aminomethane (buffer); egg yolk (organic substance); fructose (energy source); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

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Components of Egg Yolk-Tris Extender

	Glycerol:	4-8% vol/vol
	Citric Acid:	55-75 mM
	Tris [hydroxymethyl]aminomethane:	190-210 mM
5	Egg yolk:	5-25% vol/vol
	Fructose:	45-65 mM
	Tylosin:	25-100 µg/ml
	Gentamicin:	200-300 µg/ml
	Linco-spectin:	100-400 µg/ml*
10	* 100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin	

In a variation of this embodiment particularly suitable for freezing bovine sperm, the concentrations of these components after addition of the final extender to the isolated sperm are about 6% (vol/vol) glycerol, about 65 mM citric acid, about 200 mM

15 Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 56 mM fructose, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 150/300 µg/ml linco-spectin (i.e., 150 µg/ml lincomycin and 300 µg/ml spectinomycin), in deionized water.

In an alternative embodiment, an egg yolk-citrate extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); sodium

20 citrate (buffer); egg yolk (organic substance); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

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Components of Egg Yolk-Citrate Extender

Glycerol:	4-8% vol/vol
Sodium Citrate :	60-80 mM
Egg yolk:	5-25% vol/vol
Tylosin:	25-100 µg/ml
Gentamicin:	200-300 µg/ml
Linco-spectin:	100-400 µg/mL*

* 100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin

Exemplary, preferred concentrations for freezing bovine sperm are about 7% (vol/vol) glycerol, about 72 mM sodium citrate, about 20% (vol/vol) egg yolk, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

In another alternative embodiment, an egg yolk-TES-Tris ("EY TEST") extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); egg yolk and heated milk, e.g., homogenized milk containing 1.25% fructose with 10% glycerol (organic substances); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

Components of Egg Yolk TES-Tris Extender

	Glycerol:	3-7% vol/vol
	Tris [hydroxymethy-methyl]-2-aminoethanesulfonic acid:	140-170 mM
5	Tris [hydroxymethyl]aminomethane:	60-80 mM
	Egg yolk:	5-25% vol/vol
	Fructose:	5-12 mM
	Tylosin:	50-150 µg/ml
	Gentamicin:	400-600 µg/ml
10	Linco-spectin:	200-700 µg/mL *

* 200-700 µg/ml lincomycin and 200-700 µg/ml spectinomycin

Exemplary, preferred concentrations for freezing bovine sperm are about 5% (vol/vol) glycerol, about 158mM Tris[hydroxymethy-methyl]-2-aminoethanesulfonic acid, about 72 mM
15 Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 8mM fructose, about 100 µg/ml tylosin, about 500 µg/ml gentamicin, and about 300/600 µg/ml linco-spectin.

In yet another alternative embodiment of the invention, a Milk extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); heated homogenized milk (organic substance); fructose (energy source); and tylosin, gentamicin,
20 and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

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Components of Milk Extender

	Homogenized Milk	90% (vol/vol)
	Glycerol:	3-7% (vol/vol)
5	Fructose:	1.25 % (wt/vol)
	Tylosin:	50 µg/ml
	Gentamicin:	250 µg/ml
	Linco-spectin:	250/300 µg/ml

*

10 * 250-300 µg/ml lincomycin and 250-300 µg/ml spectinomycin

Exemplary preferred concentrations for freezing bovine sperm are about 90% milk, about 10% (vol/vol) glycerol, about 1.25% fructose (wt/vol?), about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

15 Other extenders standardly used to freeze sperm can also be employed as the final extender in freezing selected sperm. A variety of extenders optimized for use in freezing sperm from various species have been described, and many are commercially available. Freezing extenders for equine sperm typically consist of milk, egg yolk, various sugars, electrolytes and a cryoprotectant. Exemplary freezing extenders are described by Squires, E.L., et al., Cooled and
20 Frozen Stallion Semen Animal Reprod. and Biotechnology Laboratory, Bulletin No. 69, Chapter 8, "Seminal Extenders" pp. 49-51 (July, 1999).

Equilibration and Freezing of Sperm

Extension of the sperm sample produces a suspension of sperm, which is then transferred
25 into containers for freezing. If the sperm are intended for use in fertilization, the cells are conveniently aliquoted into individual doses sufficient to achieve fertilization. The required dose can vary substantially from one species to the next and is either well-known (e.g., for cattle and horses) or can readily be determined. In the case of sex-selected bovine sperm, convenient doses range from about 1.0×10^6 sperm to about 3.0×10^6 sperm.

30 Any suitable container can be employed for freezing, including, for example, an ampule, a vial, and a straw. Sperm intended for AI are typically frozen in straws (e.g., 0.25 ml or 0.50 ml

straws) designed for use with an insemination gun. Preferably, a bolus of extender is drawn into the straw, followed, in sequence, by air, sperm, air, and extender, so that the sperm are flanked on either side by an air space, which separates the sperm from a bolus of extender at either end of the straw.

5 Prior to freezing, the sperm are generally allowed to equilibrate at about 5°C. Preferably, the sperm are allowed to equilibrate for a period in the range of about 1 hour to about 18 hours, more preferably between about 3 hours and about 18 hours, and most preferably between about 3 hours and about 6 hours (see Example 2).

10 Following equilibration, any standard freezing method can be employed, provided the freezing rate is not too rapid (i.e., not in excess of about 0.5°C/minute). Preferably, the freezing rate is about 0.5°C/minute. In an exemplary, preferred embodiment, the sperm are placed in static liquid nitrogen vapor, and freezing is carried out in three distinct stages over a period of about 10 minutes. In the first stage of freezing, the sperm are cooled from about 5°C to about -15°C at a rate of about 40°C/minute to about 65°C/minute. In the second stage of freezing, the sperm are cooled from about -15°C to about -60°C at a rate of about 25°C/minute to about 35°C/minute. In the third stage, the sperm are plunged into liquid nitrogen at about -100°C.

Selected Sperm Samples

20 In addition to a freezing method, the invention provides a frozen sperm sample including sperm selected from a source sample for a particular characteristic. The sperm can be from any species, including any of those discussed above with reference to the freezing method. The invention encompasses frozen sperm selected for any characteristic by any suitable method, such as those described above. Preferred embodiments include frozen sex-selected human, bovine, equine, porcine, ovine, elk, or bison sperm. Sex-selection is preferably carried out using flow cytometry as described generally above.

25 Also within the scope of the invention is a container containing a frozen sperm sample according to the invention. The container can be formed from any material that does not react with the frozen sperm sample and can have any shape or other feature that facilitates use of the sample for the intended application. For samples intended for use in AI, for example, the container is conveniently a straw (e.g., 0.25 ml or 0.5 ml straw) designed for use with an insemination gun. The container is sealed in any manner suitable for preserving the sample at

the intended storage temperature, which is typically below -80°Celsius. 0.25 ml straws can be sealed, for instance, with PVC powder, ultrasonically, or with a cotton-polyvinyl plug and/or a stainless steel ball (BB).

5 As the frozen sperm sample of the invention is typically thawed before use, the invention also provides a thawed, previously frozen, selected sperm sample and a container including such a thawed sample.

Methods of Using the Selected Sperm Sample

10 The frozen selected sperm sample of the invention is suitable for use in any method in which sperm are used. The sample can be thawed and used in any conventional fertilization method, such as artificial insemination or in vitro fertilization. Thawing is carried out in the same manner as for frozen, non-selected sperm. Briefly, the straw containing the frozen sperm is submerged in a water bath maintained at a temperature of about 35°C to about 37°C for a period of about 20 to about 30 seconds. After thawing, semen deposition (e.g., insemination) is carried out according to standard procedures, taking care to protect the sperm from environmental
15 fluctuations.

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EXAMPLES

Example 1

Effects Of Dilution On Sperm

- 5 Objective: to determine the effect of sperm concentration on sperm motility for non-frozen, non-sorted, but highly diluted sperm.

A. *Effects of Dilution on Non-washed Sperm*

1. Collection of Source Sample. Sperm were collected from bulls on a routine
10 collection schedule using an artificial vagina as described in Schenk J., Proc 17th NAAB, p.48-58 (1998), and Saacke RG, Proc NAAB Tech Conf AI Reprod. 41:22-27 (1972). All ejaculates used contained greater than 50% progressively motile and greater than 75% morphologically normal sperm. Antibiotics were added to the raw ejaculate as described by Shin S., Proc NAAB Tech Conf AI Reprod. 11:33-38 (1986) within 15 minutes of collection, and the concentration of sperm was determined using a spectrophotometer.

2. Methods. Sperm from 4 bulls were diluted to 1.25, 2.5, 5, 10, 15, and 20×10^6 /ml using an egg yolk-citrate extender (EYC) prepared with 20% egg yolk (vol/vol) in 72 mM sodium citrate, 50 µg/ml tylosin, 250 µg/ml gentamicin, and 250/300 µg/ml linco-spectin. Each
20 sample was prepared in duplicate (2 tubes/dilution/bull) and comprised 8 ml total volume per tube. All samples were incubated for 60 minutes at 22°C, after which they were centrifuged using a swinging bucket centrifuge (Eppendorf, Model # 5810R) at $600 \times g$ for 10 minutes to concentrate the sperm. After centrifugation, the supernatant from one set of the duplicate tubes was not removed; the sperm were resuspended in the same medium and at the original
25 concentration by repeated gentle aspiration using a 5-ml serological pipette. (The second set of the duplicate tubes were used in Example 1B.) Sperm samples were then cooled to 5°C at 0.2°C/min over 90 minutes. These sperm were termed “non-washed sperm.” All samples were incubated at 5°C for 24 or 48 h post-collection.

3. Evaluation of Motility. After incubation, the samples were warmed to 37°C using
30 a dry block incubator for 10 minutes prior to determination of motility. For this experiment, a

single, blind estimate of the percentage of progressively motile sperm was determined for each sample. Progressive sperm motility was determined subjectively for each subclass by a single observer (x200, phase-contrast microscopy); another person prepared the microscope slides in a randomized manner so the observer was unaware of treatments.

5

4. Statistical Analysis. Data were analyzed by analysis of variance (SAS Institute, Cary, North Carolina) with factors bulls and initial dilution concentration. Separate analyses were done for each incubation time. Dilution trends were tested using (log) linear contrasts.

10 5. Results. Data for non-washed sperm (Table 1) revealed (log) linear relationships (P<0.01) for both incubation times. Percentages of motile sperm increased as sperm concentration increased from $1.25 \times 10^6/\text{ml}$ to $10 \times 10^6/\text{ml}$, but there was little difference thereafter. The cubic term was significant (P<0.05) for 24-h and marginally significant (P<0.1) for 48-h incubations. There was a bull effect (P<0.01) at both times.

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Table 1. Effects of cooling on non-washed sperm motility (%) after cooling to 5°C.

Dilution (10 ⁶ /ml)	Incubation at 5°C	
	24 h ^a	48 h ^b
1.25	18 ^c	0 ^c
2.5	38 ^{c,d}	6 ^{c,d}
5.0	56 ^d	31 ^{d,e}
10.0	61 ^d	42 ^e
15.0	55 ^d	44 ^e
20.0	58 ^d	41 ^e
S.E. ^f	5.6	6.4

^a (log) linear (P<0.01) and cubic effects (P<0.05).

^b (log) linear (P<0.01) and cubic effects (P<0.1).

^{c,d,e} Means within columns without common superscripts differ (P<0.05).

^f $\sqrt{\text{error mean square of ANOVA} - \sqrt{N}}$
(SAS Institute, Cary, NC, USA)

B. Effects of Dilution on Washed Sperm

1. Collection of Source Sample. The second set of the duplicate tubes containing samples prepared in Example 1A were used in this experiment.

2. Methods. The sperm were diluted, incubated and concentrated by centrifugation as in Example 1A. Following centrifugation, 7.1 ml of the supernatant was aspirated from each tube, removing most of the seminal plasma and leaving the sperm in a 900-μl pellet. The sperm were diluted with EYC (see Example 1A) to make 10 × 10⁶/ml or 20 × 10⁶/ml sperm suspensions. The samples were then cooled to 5°C over 90 minutes as in Example 1A.

3. Evaluation of Motility. The samples were warmed and evaluated for progressive motility as in Example 1A.

4. Statistical Analysis. Data were analyzed as in Example 1A. In addition, data in Example 1B were analyzed for incubation concentration at 5°C.

5. Results. Data for washed sperm (Table 2) revealed no significant treatment effects when sperm were evaluated after 24 h. However, after storage for 48 h at 5°C, there were bull, initial dilution, incubation concentration and bull by incubation effects ($P < 0.05$). More sperm remained motile when held at 20×10^6 /ml than at 10×10^6 /ml (31% vs. 20%; $P < 0.05$). Initial dilutions of 1.25, 2.5, and 5×10^6 sperm/ml resulted in lower progressive motility than 10×10^6 sperm/ml ($P < 0.05$), with respective main effect means of 19, 20, 27, and 37% motile sperm.

Table 2. Cumulative effects of washing, dilution, concentration and cooling on progressive sperm motility (%)

Sperm conc (10^6 /ml) during 1 h preincubation at 37°C	Storage at 5°C – Sperm Concentration and Duration			
	24 h		48 h ^a	
	20×10^6 /ml	10×10^6 /ml	20×10^6 /ml	10×10^6 /ml ^b
1.25	45	49	24	15
2.5	51	40	29	11
5.0	54	54	32	21
10.0	51	50	40	34
15.0	60		41	
20.0	55		40	

^a Concentration to 20×10^6 sperm/ml was superior ($P < 0.05$) to 10×10^6 sperm/ml after 48 h storage.

Also, initial dilution to 10×10^6 was superior to lower dilutions ($P < 0.05$).

Pooled standard errors ($\sqrt{\text{error mean square of ANOVA}} / \sqrt{N}$) were 4.0 for 24 h, and 2.8 for 48 h incubations.

^b Significant (log) linear trend ($P < 0.06$).

C. Conclusion

High sperm dilution and cooling resulted in a substantial reduction in the percentage of motile sperm, regardless of the presence or removal of seminal plasma. However, this dilution

effect was greatly attenuated by concentrating the diluted sperm to $10 \times 10^6/\text{ml}$ and even more, to $20 \times 10^6/\text{ml}$ before storage at 5°C . Sperm from some bulls tolerated dilution better than sperm from other bulls; however, the bull differences found are typical. Extremely dilute sperm might be compromised during sorting, in part, by removal of protective compounds in seminal plasma.

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Example 2

Effects of Equilibration Time Before Freezing Sorted Sperm

Objective: to evaluate the effect of equilibration times (3, 6 and 18 h, 5°C) before freezing on flow-sorted sperm.

10

The following experiment was replicated in its entirety using the same bulls:

1. Collection of Source Sample. Sperm of 4 bulls were collected and prepared as described in Example 1A.

15

2. Methods.

a) Staining and Preparation for Sort.

20

i) Preparation of Stain Stock Solution: a stock solution of 8.89 mM Hoechst 33342 (bis-Benzimide H-33342; #190305, ICN Biomedicals Inc., Aurora, OH) was prepared in deionized water.

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ii) Sperm Stain Procedure: sperm were diluted in a modified TALP buffer (Table 3) to 400×10^6 sperm/ml. Following dilution, Hoechst 33342 dye was added to the sperm suspensions at a concentration of 224 μM . After the stain was added to the sperm suspensions, the samples were incubated for 60 minutes at 34°C . Following incubation, sperm were diluted to $100 \times 10^6/\text{ml}$ with TALP containing 2.67% clarified egg yolk and 0.002% food coloring dye (FD&C #40) which quenches the fluorescence of Hoechst 33342 in sperm with compromised cell membranes, allowing them to be gated out during the sorting process. Just prior to flow sorting, samples were filtered at unit gravity through a 40- μm nylon mesh filter to remove any debris and/or clumped sperm.

30

b) Sorting. A two-line argon laser operating at 351 and 364 nm and 150 mW was used to excite the Hoechst 33342 dye. The flow cytometer/cell sorter used was an SX MoFlo[®] (Cytomation, Inc., Fort Collins, CO, USA) operating at 50 psi. A Tris-based sheath fluid was used, consisting of Tris (hydroxymethyl) aminomethane (Tris; 197.0 mM; #T-1503, Sigma Chemical Co., St. Louis, MO, USA), citric acid monohydrate (55.4 mM; #C-7129, Sigma Chemical Co., St. Louis, MO, USA) and fructose (47.5 mM; #F-0127, Sigma Chemical Co., St. Louis, MO, USA). Baseline antibiotics were also added to the Tris-based sheath fluid consisting of 0.58 g/L of penicillin and 0.05 g/L of streptomycin sulfate.

The sperm were sorted by a process referred to as “bulk sorting” which permits rapid accumulation of large numbers of sperm so that large-scale examples can be done within a reasonable time. The sperm pass through the flow cytometer under the standard operating conditions with the exception that all droplets containing viable sperm were collected into a single tube rather than being sorted into 2 tubes based upon specific characteristics (e.g., sorting by sex-type). Sperm were sorted on the basis of viability; hence, sperm that have compromised plasma membranes were excluded during bulk sorting.

Stained sperm were maintained at $22 \pm 1^{\circ}\text{C}$ during sorting. Bulk sorted sperm were collected in 50-ml plastic tubes containing 2 ml of 20% egg yolk-Tris extender prepared with 20% egg yolk (vol/vol) in 200 mM Tris, 65 mM citric acid, 56 mM fructose, 50 $\mu\text{g/ml}$ tylosin, 250 $\mu\text{g/ml}$ gentamicin, and 150/300 $\mu\text{g/ml}$ linco-spectin in deionized water. The egg yolk-Tris extender was termed “Tris-A fraction” to denote the lack of glycerol at this point in the procedure. Sperm were collected in tubes to contain 12 ml and approximately 6×10^6 sperm. The sperm were subsequently incubated at 22°C for 1 to 3 h to simulate conditions of a sort based on sex-type.

c) Preparation for Freezing. Following incubation, the sorted sperm were cooled to 5°C over the period of 70 minutes. After cooling, the contents of the two tubes were pooled and transferred to a refrigerated, swinging bucket centrifuge set at 5°C and

centrifuged at $850 \times g$ for 20 minutes. After removing the supernatant, processing continued at 5°C by adding about 150 μl of Tris-A fraction extender to about 150- μl of sperm pellet to bring the sperm concentration to approximately $40 \times 10^6/\text{ml}$. The sperm of individual bulls were pooled and diluted immediately with an equal volume of egg yolk-Tris extender containing 12% (v/v) glycerol ("Tris-B fraction"). The Tris-B fraction was added to the sperm suspension as 2 equal volumes at 15-minute intervals to adjust the final sperm concentration to $20 \times 10^6/\text{ml}$. The final glycerol concentration of the complete egg yolk-Tris extender containing the sperm was 6% (v/v).

d) Equilibration and Freezing. Extended sperm were then packaged into 0.25-ml polyvinylchloride straws to be frozen by routine procedures on racks in static liquid nitrogen vapor. Two straws from each of 4 bulls were frozen after 3, 6 and 18 h of total equilibration time at 5°C .

3. Evaluation of Post-Thaw Motility. Straws were thawed in a 37°C water bath for 30 sec. Blind estimates of progressive motility were made after incubating samples at 37°C for 0, 1 and 2 h post-thawing. Each of two observers estimated progressive sperm motility from each of two straws of semen. These four blind estimates for each experimental unit represent subsampling.

4. Statistical Analysis. Statistically, the subsamples were analyzed as a subplot to the main plot least-squares ANOVAs to analyze effects of any observer and observer x treatment interaction. N refers to the number of experimental units, not subsamples; standard errors were calculated on the basis of means of the 4 subsamples from error mean squares of ANOVAs and the numbers of experimental units; least-squares means are presented.

Treatment effects were evaluated via separate ANOVAs for each incubation time. The model included bulls as a random effect and equilibration time and observer as fixed effects; the subplot consisted of the observer term and related interactions.

5. Results. The 3- or 6-h equilibration times were superior to 18-h (Table 4), based on the percentage of progressively motile sperm, for 0 and 1 h ($P<0.01$) but not 2 h of post-thaw incubation. Effects of bull were evident at 1 and 2 h incubation times ($P<0.05$), but not at 0 h. There was no significant ($P>0.1$) bull by equilibration time interaction nor was there a significant observer effect for any response.

Table 3. Modified TALP Buffer

Ingredient	Concentration
NaCl	95.0 mM
KCl	3.0 mM
NaHPO ₄	0.3 mM
NaHCO ₃	10.0 mM
MgCl ₂ • 6H ₂ O	0.4 mM
Na Pyruvate	2.0 mM
Glucose	5.0 mM
Na lactate	25.0 mM
HEPES ^a	40.0 mM
Bovine serum albumin ^b	3.0 mg/ml
Gentamycin Sulfate	30.0 µg/ml

^a #H3375, Sigma Chemical Co., St. Louis, MO, USA

^b #US70195, fraction V; Amersham/Life Science, Cleveland, OH, USA

Table 4. Effect of pre-freeze equilibration time on post-thaw progressive motility (%)

Equilibration at 5°C	Post-thaw incubation at 37°C		
	0 h	1 h	2 h
3 h	41 ^a	36 ^{a,b}	16
6 h	41 ^a	37 ^a	18
18 h	35 ^b	31 ^b	12
S.E. ^c	1.5	0.8	2.0

^{a,b} Within columns, means without common superscripts differ (P<0.05), Tukey's HSD.

^c Pooled standard errors,

$$\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$$

6. Conclusion. The results indicated no differences in post-thaw sperm motility between 3 and 6 h of total equilibration time at 5°C, but there was a significant decline in sperm motility following 18 h of equilibration at 5°C before freezing. The 3- to 6-h range permits pooling 2 consecutive 3-h sorting batches for freezing sperm without decreasing post-thaw motility.

As the bull by equilibration-time interaction was not significant, 3 to 6 h equilibration was adequate, with the caveat that only 4 bulls were used. The optimum equilibration time for a minority of bulls is expected to be >6 h.

Example 3

Effects Of Stain Concentration And Laser Power on Sorted Sperm

Objective: to evaluate the effects of Hoechst 33342 dye concentration in combination with laser intensity on flow-sorted sperm.

5

1. Collection of Source Sample. Sperm of 6 bulls were collected and prepared as described in Example 1A.

2. Methods.

10

a) Experimental Design. One ejaculate (2 bulls) and 2 ejaculates on different days (4 bulls) were used in a 2 by 2 design plus control.

15

b) Staining and Sorting. Staining, preparation for sorting and sorting sperm were achieved as described in Example 2 except that the Hoechst 33342 dye was added to sperm suspensions at a final concentration of 149 μM or 224 μM ; and sperm were bulk-sorted with the laser operating at 100 mW or 150 mW of incident power. Bulk-sorted sperm were collected into 50-ml plastic tubes as described in Example 2. Four tubes containing approximately 15×10^6 total sperm/tube were collected over 1 h for each bull. The sorted sperm were incubated for 1 h at 22°C to simulate a longer sorting time.

20

c) Preparation for Freezing. Following incubation, the sperm were cooled as in Example 2. The sperm were then concentrated by centrifugation at 5°C at $850 \times g$ for 20 minutes. After removing the supernatant, 150 μl of Tris-A fraction extender was added to each 150- μl sperm pellet at 5°C. All of the sperm pellets were suspended by gentle repeated aspiration and the sperm of individual bulls were pooled. Tris B-fraction extender was added stepwise as described in Example 2. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in Tris extender containing 6% glycerol and cooled to 5°C while the bulk-sorted sperm were being prepared.

25

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d) Equilibration and Freezing. The control and sorted sperm were packaged into 0.25-ml polyvinylchloride straws as described in Example 2, equilibrated at 5°C for 3 h and then frozen conventionally.

5 3. Evaluation of Post-Thaw Motility. Straws were thawed and evaluated as described in Example 2.

 4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via ANOVA. The model included dye
10 concentration, laser intensity and bulls in the main plot, and observer and related interactions in the subplot. Bulls were considered a random effect and the other factors as fixed.

 5. Results. Bull effects were significant for percentages of progressively motile sperm immediately after thawing ($P<0.1$) and after 1 h and 2 h of incubation at 37°C ($P<0.05$).
15 There was no effect of dye concentration or bull by dye concentration on sperm motility at any incubation time. With bulls considered as a random effect, 150 mW of laser power resulted in lower post-thaw motility of sperm than 100 mW at 0 h of incubation ($P<0.1$), but not at other incubation times (Table 5). If bulls are considered as fixed effects, 150 mW of power resulted in lower sperm motility than 100 mW ($P<0.05$) at all 3 incubation times. There was an effect of
20 bull by laser power ($P<0.05$) on sperm motility at 1 h, but not at 0-h or 2-h incubation times. Also, the higher laser power resulted in lower sperm motility than the control ($P<0.05$) at 0- and 1-h incubation times (Table 5). There was a significant observer effect at 1-h, but not at 0-h or 2-h, incubation times. There was no observer by treatment interaction ($P>0.1$).

25

30

Table 5. Effects of laser intensity and dye concentration on post-thaw motility (%).

Main effect means	Incubation at 37°C		
	0 h	1 h	2 h
Control	49	44	33
<u>Dye Concentration</u>			
149 µM	41	39	30
224 µM	42	39	30
<u>Laser Intensity</u>			
100 mW	46	42	33
150 mW	38 ^a	35 ^b	27
S.E. ^c	2.2	1.2	1.3

^a Significant main effect (P<0.1) and differs from control (P<0.05).

^b Differs from control (P<0.05).

^c Pooled standard errors, $\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$

6. Conclusion. Percentages of progressively motile sperm post-thaw were diminished by the staining and sorting process. Higher laser intensity was more damaging than the lower laser intensity. There was no effect of dye concentration on post-thaw sperm motility. Thus, excitation of the sperm-bound Hoechst 33342 dye at lower laser intensities is less damaging and that staining sperm at the higher dye concentration had no detrimental effect on post-thaw motility. The damage observed was presumably to the sperm-motility apparatus.

Example 4

Evaluation of Pre-Sort Staining Procedures and Selection of Extenders for the Cryopreservation Of Sperm

Objective: (1) to evaluate three pre-sort treatments for sperm; and, (2) and to evaluate sheath
5 fluid and extender combinations for the cryopreservation of flow-sorted sperm.

The following experiment was replicated in its entirety:

1. Collection of Source Sample. Sperm from 4 bulls were collected and prepared as
described in Example 1A.

10

2. Methods.

a) Experimental Design. A 3 (pre-sort treatments) by 3 (extenders) by 2
(sheath fluids) by 4 (bulls) by 2 (observers) factorial experiment was designed to
determine the best procedure to hold sperm prior to sorting, and to evaluate three
15 extenders for cryopreserving the sorted sperm. .

15

b) Sample Preparation and Staining. Freshly collected sperm from each of 4
bulls were treated as follows:

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(1) diluted to 400×10^6 /ml in modified TALP (see Example 2, Table 3)
and stained for 1 h at 34°C before bulk-sorting (“Dilute – 0 h”);

(2) incubated neat at 22°C for 3 h before dilution, staining and sorting
 (“Neat – 3 h”); or,

25

(3) diluted and stained as “Dilute-0 h” and then incubated at 22°C for 3 h
before bulk-sorting (“Diluted – 3 h”).

30

c) Extenders. The following freezing extenders were compared: EYC (see
Example 1) containing 7% glycerol, egg yolk-Tris (see Example 2) containing 6%
glycerol, and egg yolk-TES-Tris (TEST) containing 5% glycerol. EYC “A
Fraction” refers to the EYC extender containing no glycerol, and EYC “B
Fraction” refers to EYC extender containing twice the final, desired glycerol

concentration (i.e., 14%). Thus, when EYC A and B fractions are combined in equal volume, the final EYC extender contains 7% glycerol. Tris A and B fractions are similarly named, and described in Example 2. TEST extender is prepared as a complete extender containing 5% glycerol; hence, there were no “A” and “B” fractions for TEST.

d) Sheath Fluid. Sheath fluid was either 98.6 mM sodium citrate dihydrate (#S279-3, Fisher Scientific, Fair Lawn, NJ) or Tris as described in Example 2. Both types of sheath fluid were adjusted to pH 6.8; osmolality was about 270 to 280 mOsm/kg. Tris sheath fluid was used to collect sperm that were later extended in egg yolk-Tris and TEST freezing extenders. Sheath fluid containing 98.6 mM sodium citrate dihydrate was used to collect sperm to later be extended in EYC freezing extender.

e) Sorting. Approximately 58×10^6 sperm for each combination of pre-sort treatment, sheath fluid and extender were bulk-sorted as described in Example 2 using 150 mW of incident laser power. For each sort, sperm were collected over approximately 1 h. After sorting, the samples were incubated at 22°C for 2 h to simulate a 3 h sort.

f) Preparation for Freezing. Following incubation, the sperm were cooled as described in Example 2. After cooling, the samples were centrifuged at 5°C at $850 \times g$ for 20 min. Each sample comprised about 28 ml total volume and was contained in a 50-ml plastic tube

After the supernatant was removed, the sperm were returned to a 5°C cold room for extension. Samples were extended to 40×10^6 /ml by depositing 131 µl of the sperm suspension into 69 µl of A-fraction EYC, A-fraction egg yolk-Tris, or TEST extender. Immediately, suspensions were adjusted to 20×10^6 sperm/ml with the addition of the matched glycerol containing extender (i.e., B-fraction EYC, B-fraction Tris) or TEST. B-fraction extenders were added to their respective samples stepwise (2X) at 15-min intervals as described in Example 2.

The TEST was added to sperm stepwise in the same manner as B-fraction EYC and Tris extenders.

g) Equilibration and Freezing. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for 3 h at 5°C and then frozen in static liquid nitrogen vapor.

3. Evaluation of Post-Thaw Motility. Thawing and post-thaw evaluations of sperm were done as described for Example 2.

4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each post-thaw incubation time. The main plot included pre-sort treatment, extenders, and bulls; the subplot consisted of observers and associated interactions. Bulls were considered a random effect, and the other factors, fixed. The entire experiment was replicated twice. Tukey's HSD test was used to separate means.

5. Results. Post-thaw progressive motility of bulk-sorted sperm was affected ($P < 0.05$) by extender and bulls at each post-thaw incubation time and by pre-sort procedure at 0 h of incubation (Table 6). There were no differences due to sheath fluids ($P > 0.05$). At 0-h post-thaw incubation, use of the neat-3h treatment resulted in more motile sperm after freezing and thawing than the other 2 pre-sort staining treatments ($P < 0.05$; Table 6). However, pre-sort procedures were not statistically significant after post-thaw incubation of sperm for 1 or 2 h with bulls considered as a random effect. Importantly, at these 2 incubation times, there were significant pre-sort treatment by bull interactions ($P < 0.05$). Furthermore, pre-sort treatment would have been a significant effect at all post-thaw incubation times had bulls been considered as fixed effects.

Immediately after thawing (0 h), TEST was the best extender, but after 1 or 2 h of incubation of 37°C, Tris was the best extender. Importantly, there was no pre-sort treatment by extender interaction for any response. There were observer effects ($P < 0.01$) at all incubation

times, but no observer by treatment interactions. There was a bull by extender interaction (P<0.05) at all 3 incubation times.

Table 6. Main effects of pre-sort treatment and freezing extenders on post-thaw progressive motility (%)

Pre-sort procedure	Extender	Incubation at 37°C		
		0 h	1 h	2 h
Dilute – 0 h	Mean	39 ^a	32	22
Neat – 3 h	Mean	43 ^b	36	25
Dilute – 3 h	Mean	38 ^a	31	19
Mean	EYC	36 ^a	29 ^a	17 ^a
Mean	Tris	40 ^b	39 ^b	29 ^b
Mean	TEST	44 ^c	33 ^c	20 ^a
S.E. ^d		0.8	0.8	0.7

^{a,b,c} Means within columns, within main effects, without common superscripts differ (P<0.05).

^d Pooled standard errors = $\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$

6. Conclusion. This study showed that holding sperm neat for 3 h before dilution, staining and sorting was better than immediate dilution and staining 0 h or 3 h later. Thus, by 3 h into the sort, it is best to continue with a new aliquot of the original ejaculate that was held neat 3 h and then stained, rather than continuing with the original sample of sperm stained and held at 400 x 10⁶ sperm/ml.

Even though TEST extender provided higher post-thaw motility at 0 h, Tris was the superior extender when sperm were stressed by incubation at 37°C. Either sheath fluid worked equally well for each extender. Based on these results, we have incorporated the use of Tris sheath fluid in combination with Tris freezing extender into our standard operating procedure.

Example 5

Effects Of Extender Additives on Sorted Sperm

Objective: to evaluate the effect of adding sodium dodecyl sulfate ("SDS") to the freezing extender on flow-sorted sperm.

5

A. *Evaluation of Effect of Concentration of SDS in Freezing Extender*

1. Collection of Source Sample. Sperm of 6 bulls were collected and prepared as described in Example 1A.

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2. Methods. Sperm from each of 6 bulls were extended to 20×10^6 /ml in 20% whole egg Tris ("WET") extender containing 0, 0.03, 0.06, 0.09, or 0.12 percent SDS, packaged into straws and frozen. WET extender was prepared using 3.028 g of Tris[hydroxymethyl] aminomethane, 1.78 g of citric acid monohydrate, and 1.25 g of fructose per 100 ml of double distilled water, to which 20% whole egg (vol/vol) was added. The WET extender was prepared at a pH of about 7.0 and contained a final glycerol concentration of about 6% (vol/vol). The WET extender also contained 1000 IU of penicillin "G" sodium and 100 µg of streptomycin sulfate/ml.

15

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3. Results. The respective means (n = 1 sample from each of 6 bulls) were 51, 51, 50, 51, and 48% progressive motile sperm approximately 10 minutes post-thaw. Based on these results, 0.06 percent SDS was used in Example 5B.

B. *Evaluation of the Effects of 0.06 Percent SDS in Various Freezing Extenders on Post-Thaw Motility of Flow-Sorted Sperm*

25

1. Collection of Source Sample. Sperm of 8 bulls were collected and prepared as described in Example 1A.

30

2. Methods. Post-thaw motility was studied for sperm frozen in egg yolk-Tris (see Example 2) and WET extenders (see Example 5A) with and without 0.06% SDS. Final glycerol content for both extenders was 6%.

a) Staining, Preparation for Sort, Sorting. Stained sperm samples were prepared from an ejaculate from each of 8 bulls as described in Example 2. Stained sperm were bulked-sorted using Tris sheath fluid as described in Example 2 except that the sort was achieved using 135 mW of incident laser power. Sorted sperm were collected in a 50-ml plastic tube containing 2 ml of A-fraction freezing buffer for each extender; 15×10^6 total sorted sperm (25 ml) for each treatment were collected and incubated for 1 h at 22°C to simulate longer sorting.

b) Preparation for Freezing. Diluted sperm were then cooled to 5°C over 90 minutes. An equal volume of appropriate B-fraction extender was added stepwise (2x) at 15-minute intervals to each 50-ml plastic tube containing sorted sperm. Aliquots of 25 ml/extender treatment were concentrated by centrifugation for 20 minutes at 850 x g in a refrigerated centrifuge. The supernatant was removed leaving a 600 µl sperm pellet, which was suspended by gentle vortexing for 15 seconds. No additional extender was added to the sperm pellet since the suspension containing the pellet already contained glycerol. The concentration of the sperm suspension was approximately 20×10^6 /ml. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in egg-yolk-Tris extender containing 6% glycerol. The control was placed in a 5°C cold room while bulk-sorting occurred.

c) Equilibration and Freezing. All control and bulk-sorted sperm were packaged and frozen at the same time. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for about 3 h to about 6 h at 5°C and then frozen in static liquid nitrogen vapor.

3. Evaluation of Post-Thaw Motility. Thawing and post-thaw evaluations of sperm were done as described for Example 2 with the exception that progressive motility was evaluated 0.5 and 2.0 h after incubation.

4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each incubation time; the model included bull and extender in the main plot and observer and

related interactions in the subplot. Differences in means were determined by the least significant difference test.

5. Results. Extender affected ($P<0.05$) progressive motility of sperm after 0.5 or 2 h post-thaw incubation (Table 7). At 0.5 h, WET plus SDS resulted in lower motility than Tris with SDS. At 2 h, all treatments with bulk-sorted sperm were worse than the non-sorted control sperm. There were significant bull and observer effects ($P<0.01$) at both incubation times, but no observer by treatment interactions.

Table 7. Effect of extender on post-thaw progressive motility (%)

Extender	Incubation at 37°C	
	0.5 h	2 h
Tris (non-sort)	42 ^a	41 ^a
Tris w/o SDS	40 ^{a,b}	35 ^b
Tris w/SDS	42 ^a	37 ^b
WET w/o SDS	40 ^{a,b}	35 ^b
WET w/SDS	38 ^b	35 ^b
S.E. ^c	1.0	1.2

^{a,b} Means within columns without common superscripts differ ($P<0.05$).

^c $\sqrt{\text{error mean square of ANOVA}} \div \sqrt{N}$

6. Conclusion. The inclusion of SDS in Tris or WET extenders did not benefit sperm quality as determined by visual estimates of motility after thawing. Also, results using WET and Tris extenders were similar; hence, WET appeared as efficacious as Tris for cryopreserving sorted bovine sperm.

Example 6

Quality of Sperm Sexed by Flow Sorting for Field Trials

Objective: to evaluate post-thaw quality of sorted sperm based on acrosomal integrity.

5 1. Collection of Source Sample. Sperm of 3 bulls were collected and prepared as described in Example 1A.

 2. Methods. Sorted and non-sorted control sperm from the same ejaculate were stained, processed, and sorted as described in Example 2 except the sperm were sorted for sex-
10 type at a 90% purity level. Sorted sperm were collected to a volume of approximately 20 ml and were cooled to 5°C for 90 minutes (0.2°C/min). After cooling, an equal volume of egg yolk-Tris B extender (see Example 2) was added to the sorted sperm in 2 equal volumes at 15-minute intervals. Centrifugation and aspiration of the supernatant were achieved as described in Example 5. After centrifugation and aspiration, egg yolk-Tris extender containing 6% glycerol
15 (v/v) was added to the sperm pellet to bring the concentration of sperm to about 20×10^6 /ml. Freezing and thawing were done as described in Example 2 except that equilibration time was about 3 h.

 3. Evaluation of Post-Thaw Motility. Visual estimates of the percentage of
20 progressively motile sperm at 37°C were made approximately 10 minutes after thawing. The acrosomal integrity of sperm was assessed using differential interference-contrast microscopy (x1000) after 2 h of incubation at 37°C. Sperm were treated with 40 mM sodium fluoride, a wet was smear made, and 100 sperm per treatment were examined. Acrosomes were classified as: (a) intact acrosome, (b) swollen or damaged acrosome, or (c) missing acrosome (non-intact).

25 4. Statistical Analysis. The data analyzed were from 19 different freeze dates balanced across 3 bulls used in field trials. Treatment effects (sort vs. control) were evaluated via analysis of variance with bulls as a fixed effect.

 5. Results. The percentage of progressively motile sperm post-thaw was
30 significantly higher ($P < 0.05$) for non-sorted sperm (50%) than for sorted sperm (46%; Table 8), despite removal of dead sperm during sorting. However, the percentage of sperm with an intact

acrosome was not different. Sorting increased the percentage of sperm missing an acrosome, but also reduced the percentage of sperm with a damaged acrosome, relative to control sperm ($P<0.05$). There were significant differences among bulls for percent of intact acrosomes ($P<0.05$), percent of non-intact acrosomes ($P<0.01$), and post-thaw progressive motility ($P<0.01$). There was a bull by sorting effect for post-thaw motility ($p<0.01$) but not for the other responses. From bulls A and B, differences in post-thaw motility between sorted and unsorted sperm were near zero; for bull C, sorted sperm were 10 percentage points (19%) lower in motility than control sperm.

Table 8. Effect of sorting on post-thaw motility (%) and acrosomal status (%).

	Acrosomal status			Post-thaw motility
	Intact	Damaged	Non-intact	
Control	64 ^a	20 ^a	15 ^a	50 ^a
Sorted	65 ^a	14 ^b	21 ^b	46 ^b

^{a,b} Column means with different superscripts differ ($P<0.05$).

6. Conclusion. Visual estimates of progressive motility for sorted, frozen sperm on average were slightly lower (4 percentage points; 8%) than for control sperm, although this difference was larger for one bull. These evaluations were made approximately 10 minutes after thawing. The small average difference is consistent with that for non-intact acrosomes after 2 h of incubation. Sperm with a damaged or missing acrosome are likely to be immotile. The increased percentage of sperm with a non-intact acrosome, for sorted samples, indicates damage associated with sorting or with cryopreservation before or after actual sorting. Presumably, sorting converted damaged acrosomes to missing acrosomes. Based on standard procedures for evaluation of sperm quality, there is no basis for assuming that fertilizing potential of these flow-sorted sperm should be severely compromised for most bulls.

Example 7

Sex-Selection and Cryopreservation of Bull Sperm Using 20% Egg Yolk-Tris Extender

Objective: to provide a protocol for the cryopreservation of flow-sorted bull sperm.

- 5 1. Collection and Ejaculate Assessment. Collect and prepare ejaculates as described in Example 1A. Select ejaculates from those bulls with >75% morphologically normal sperm. Visually estimate the percentage of progressively motile sperm (ejaculates that have progressive motility >60% are best for sorting). Add antibiotics to raw semen as follows: tylosin at a final concentration 100 µg/ml, gentamicin at a final concentration of 500 µg/ml, and linco-spectin at a
10 final concentration of 300/600 µg/ml.

2. Staining and Preparation for Sort. Following the addition of the antibiotics to the raw semen sample, allow 15-20 minutes before staining. Stain samples as described in
15 Example 2.

3. Sorting. Sort for both X- and Y- type sperm, setting the sorting gates for 90% purity. Sort sperm into 50-ml Falcon tubes containing 2 ml 20% egg yolk-Tris A-fraction extender (see Example 2) until each tube contains a maximum of 20 ml total volume (or a maximum of 2 h per sort) and final sorted sperm concentration is 6×10^5 /ml. Note that
20 additional 20% egg yolk-Tris-A fraction catch buffer must be added after the sort and prior to cooling so that the final percentage of egg yolk is at least 3%.

4. Preparation for Freezing. Following the sort, cool the sorted samples to 5°C over a period of 90 minutes. After cooling, add 20% egg yolk-Tris B-fraction extender (see Example
25 2) stepwise (2X) at 15 minutes intervals. The final volume of Tris B-fraction extender added to the sperm sample should be equal to the volume of Tris A-fraction extender. The total volume of sperm sample after the Tris B-fraction extender is added should not exceed 27 ml total volume.

30 After the Tris B-fraction extender is added to the sperm sample, concentrate the sample by centrifugation for 20 minutes at $850 \times g$. Aspirate the supernatant leaving approximately 150 µl sperm pellet. Resuspend the sperm and pool the sperm for each individual bull.

5. Freezing. Add complete egg yolk-Tris extender (6% glycerol) to achieve a final sperm concentration of 20×10^6 /ml. Package the extended sperm into 0.25-ml polyvinylchloride straws for freezing as described in Example 2.

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Example 8

Evaluation of the Fertility of Flow-sorted, Frozen Bull Sperm in Field Studies

MATERIALS AND METHODS

10 Semen Collection and Processing

Semen from young bulls of unknown fertility was collected via artificial vagina (see Example 1A). After determining sperm concentration with a spectrophotometer and subjective evaluation of progressive sperm motility, semen was processed and sorted as described in Example 2 except that the sperm were sorted by sex-type at 90% purity using a laser incident power of about 135 to about 150 mW. Processing and freezing was achieved as in Example 2 except that the equilibration time was about 3 h. Cornell Universal Extender (Seidel GE Jr., Theriogenology 1997; 48:1255-1264) was used for liquid semen in field trials 1, 2, and 3. For frozen semen in field trials 2 and 3, the extender used was 2.9% Na citrate + 20% egg yolk with a final glycerol concentration of 7% (see Example 1). For field trials 4 through 11, sperm were frozen in a Tris-based extender composed of 200 mM Tris, 65 mM citric acid, 56 mM fructose, 20% egg yolk, and a final glycerol concentration of 6% (see Example 2). The sheath fluid used in the flow cytometer was 2.9% Na citrate (see Example 4) for trials 1, 2, and 3, and a Tris buffer for the remaining trials (see Example 2).

Sperm were packaged in 0.25-ml French straws in columns as small as 50 μ l in the center of the straw. To minimize dilution effects, low volumes were used so there were at least 10^7 sperm/ml. In most trials, a column of extender without sperm was aspirated into the straw first to wet the cotton plug, followed by a small column of air, and then the sexed sperm. When sperm were frozen, one straw from each batch was thawed in 35°C water for 30 sec for quality control, and batches with less than 25% progressive motility post thaw were discarded. A sample of sexed sperm from each batch was sonicated and analyzed by flow cytometry to determine the accuracy of sexing.

Heifer Management and Artificial Insemination

The heifers used were in 6 widely scattered production units with different management practices. Seasonal and breed differences contributed further to the heterogeneity of the experiments (Table 9). Insofar as possible, treatments and controls were alternated systematically within bulls within inseminators as heifers entered the insemination facilities.

Estrus was synchronized in one of 4 ways (Table 9): (1) 500 mg of melengesterol acetate (MGA) fed daily in 2.3 kg of grain for 14 days followed by an i.m. injection of 25 mg prostaglandin F₂α (Lutalyse, Upjohn, Kalamazoo, MI, USA) 17, 18 or 19 days after the last day of feeding MGA (MGA/PG); (2) a single injection of 25 mg of prostaglandin F₂α (PG); (3) 20 or 25 mg of prostaglandin F₂α injected i.m. at 12-day intervals (PG/PG) or (4) 50 or 100 µg of GnRH injected i.m., followed by 25 mg of prostaglandin F₂α 7 days later (GnRH/PG).

Heifers were inspected visually for standing estrus mornings and evenings, but inseminated only in the evenings after 16:00, approximately ½ or 1 day after onset of estrus. Insemination was either into the uterine body conventionally, or half into each uterine horn using atraumatic embryo transfer sheaths (IMV, Minneapolis, MN, USA). In the latter case, semen was deposited past the greater curvature of the uterine horn as far anterior as could be accomplished without trauma, identically to nonsurgical embryo transfer. In most cases, semen was deposited between the anterior third and mid-cornua.

Most experiments included a frozen sperm control inseminated into the uterine body with 20 or 40 x 10⁶ sperm/dose from the same bulls used for sperm sorted for sex-type (“sexed”). This control served as a composite estimate of the intrinsic, normal fertility of the heifers under the specific field-trial conditions as well as the fertility of the bulls used and the skills of the inseminators. Some trials also included a low-dose, unsexed control group. Sometimes numbers of control inseminations were planned to be ½ or ⅔ the number used for each treatment to obtain more information on sexed sperm. Frozen sexed and control sperm were thawed for 20 to 30 sec in a 35 to 37°C water bath. Various other details are summarized in Table 9.

Pregnancy was diagnosed by ultrasound 28 to 37 d post insemination and/or 56 to 92 d post-insemination, at which time fetal sex was determined in most trials, as described in Curran, S., Theriogenology 1991; 36:809-814, without the operator's knowing insemination treatments or controls. Sexes of calves born were nearly identical to the fetal-sex diagnosis. Data were

analyzed by single-degree-of-freedom Chi square corrected for continuity; 2-tail tests were used unless 1-tail is specified. Fewer than 5% of the inseminations were culled due to errors of insemination treatment, frank infection of the reproductive tract, failure to traverse the cervix, etc. Decisions to cull animals from experiments were made shortly after insemination and were
5 never based on the pregnancy diagnosis.

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Table 9. Procedural details of field trials

	Trial	Insemination dates	Breeds of heifers	Bulls used	Inseminator s	Estrus synchroni- zation	Comments
5	1	5/20-23, 1997	Angus	N1, N2, AN4	A, B	MGA/PG	Included low-dose controls
	2	2/18-5/22, 1998	Angus crossbred	N3, N4, N5, N6	C, D	PG/PG	Low dose but no normal- dose controls; some heifers pregnant and aborted when synchronized
10	3	6/2-6/5, 1998	Angus	AN4, AN5, N7, N8	B, D	MGA/PG	
	4	2/10-13, 1999	Holstein	J2, J4	C, D	PG	Very severe mud, snow, wind, and cold, driving rain
15	5	2/24-26, 1999	Holstein	J2, J4, J5	C, B, D	PG/PG	
	6	4/14-16, 1999	Holstein	J2, J3, J4, J5	C, D	PG	Some heifers were reproductive culls
20	7	4/27-5/1, 1999	Hereford& Angus crossbred	AN1, AN4	C	MGA/PG	Semen for 1 bull shipped 6 h before sorting; severe weather
	8	4/21-23, 1999	Angus crossbred	H1, H2	E	MGA/PG	Feedlot heifers
25	9	5/5-8, 1999	Red Angus	AR1, AR2	C, F	MGA/PG	
	10	5/31-6/2, 1999	Angus	AN4, AN7, AN8	B, D	GnRH/ PG	
	11	7/28-30, 1999	Holstein	H2, H3	C, D	PG/PG	First replicate available in a much larger trial
30							

RESULTS AND DISCUSSION

The data presented are from 11 consecutive, heterogeneous field trials, constrained by logistical aspects of the studies, such as having to match bulls to genetic needs of the herds, unavailability of fertility information on bulls, limited numbers of heifers, unavailability of the same inseminators across trials, severe weather in some trials, limited amounts of sexed semen in early trials, 2 sets of heifers in which some turned out to be pregnant up to about 55 days at the time of estrus synchronization, etc. Up to 4 bulls and 3 inseminators were involved with each trial; this enabled us to sample populations to ensure that results applied to more than one bull or technician; however, insufficient data were produced to evaluate bull-to-bull differences in fertility rigorously.

Most sets of heifers were from breeding herds located 140 to 250 km from our laboratory. There were no significant differences in pregnancy rates between inseminators in any trial, but numbers of breedings per inseminator were low, and differences likely would be detected with larger numbers of inseminations.

Estrus synchronization methods were not compared within trials, so it was not possible to compare pregnancy rates among these methods. Pregnancy rates appeared to be satisfactory for all four synchronization procedures used.

Since inseminations were done once a day, heifers in estrus evenings were inseminated approximately 24 h after estrus was detected. The pregnancy rate for these heifers with sexed sperm pooled over all trials was 203/414 (49.0%), which was not significantly different ($P>0.1$) from that of heifers in estrus mornings and thus inseminated half a day after estrus detection 266/586 (45.4%). This tendency for higher fertility with later insemination is in agreement with findings from other research that it is preferable to inseminate later than normally recommended with lower fertility bulls, when low sperm numbers are used, or when conditions are otherwise suboptimal.

Pregnancy rates by treatments and, when available, fetal or calf sex are presented in Tables 10 to 20. The objective was to obtain female offspring, except in trial 8; accuracy was 95%, 83%, 90%, 83%, 82%, and 94% in Trials 1, 3, 8, 9, 10, and 11, respectively. In the remainder of the trials, fetal or birth sexes were not available because of timing of pregnancy diagnosis, unavailability of persons skilled in sexing fetuses, and/or because calves have not yet

been born. This was not a major concern because the main objective of this research was to determine fertility of flow-sorted sperm inseminated at low doses.

The accuracy of sexing can be adjusted to virtually any level desired between 50 and 95+% by adjusting the sorting parameters. However, higher accuracy results in lower numbers of sperm sorted per unit time, particularly for Y-chromosome sperm. 90% accuracy is sufficient for routine work.

The main findings from each field trial will be summarized in turn. Note that total sperm numbers are given in table headings; numbers of progressively motile sperm usually were 30 to 50% of these values. Field trial 1 (Table 10) confirmed that pregnancy rates with uterine horn insemination using low numbers of unsexed sperm were similar to controls with normal sperm numbers. The day 64 to 67 pregnancy rate with unfrozen sexed sperm (42%) was 12 percentage points below the unsexed liquid control with sperm diluted, stained, and centrifuged identically to the sorted sperm. Accuracy of sexing was 95%; the sex of calves born from sexed sperm matched the sex diagnosis of fetuses exactly; there was one mistake in sexing fetuses of controls. There were no abortions between 2 months of gestation and term, and all 19 calves from the sexed sperm treatment were normal and survived. For the sexed semen treatment, the 2-month pregnancy rates for bulls N1, N2, and N3 were 41, 44, and 40%, respectively; 39% (13/33) of heifers in estrus in the morning and 50% (6/12) in estrus in the evening became pregnant.

Table 10. Results of field trial 1 -- Angus heifers in Wyoming, 1997

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 33	No. pregnant day 64 to 67	No. ♀ calves
Sexed, 5°C/horns	3 x 10 ⁵	45	20 (44%)	19 (42%)	18 (95%) ^a
Control, 5°C/horns	3 x 10 ⁵	28	15 (54%)	15 (54%)	5 (53%) ^b
Frozen, control/body	40 x 10 ⁶	29	16 (55%)	15 (52%)	11 (73%) ^{a,b}

^{a,b} Sex ratios without common superscripts differ (P<0.02).

Field trial 2 (Table 11) provided the first evidence that results with sexed, frozen sperm are similar to sexed, unfrozen sperm if adjustment is made for numbers of sperm killed during cryopreservation. There also was no difference in pregnancy rates between sexed sperm stored at 5 versus 18°C. Pregnancy rates at 2+ months after insemination for sexed semen from individual bulls ranged from 22 to 42% pregnant ($P>0.05$). Embryonic loss between 1 and 2 months of gestation was very similar for sexed and control pregnancies. Calving data were available from 39 heifers from this trial; each of these heifers (30 sexed pregnancies, 9 controls) pregnant at 2 months calved after a normal-length gestation.

Table 11. Results of field trial 2--Crossbred beef heifers in Colorado, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 35 ^a	No. pregnant day 59 to 92 ^a
Control, 5°C/horns	5×10^5	58	27 (47%)	24 (41%)
Sexed, 5°C/horns	5×10^5	51	17 (33%)	16 (31%)
Sexed, 18°C/horns	5×10^5	46	16 (35%)	12 (26%)
Sexed, frozen/horns	1×10^6	87	29 (33%)	28 (32%)

^a No significant differences, χ^2 .

Field trial 3 (Table 12) confirmed that sexed, frozen sperm results in reasonable pregnancy rates. The accuracy of sexing sperm was confirmed again; however, there were 4 errors in sexing fetuses relative to the calves born; the actual sexes of calves born are presented. Again, there were no abortions between 2 months of gestation and term. Pregnancy rates averaged over sexed, unfrozen and sexed, frozen sperm for bulls N8, N9, AN5, and AN4 were 24, 31, 50, and 60%, respectively ($P<0.1$).

Table 12. Results of field trial 3 -- Angus heifers in Wyoming, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 62 to 65	No. ♀ calves
Sexed, 18°C/horns	5 x 10 ⁵	37	11 (30%) ^a	10 (91%) ^c
Sexed, frozen/horns	1 x 10 ⁶	35	18 (51%) ^{a,b}	14 (78%) ^c
Frozen, control/body	40 x 10 ⁶	37	27 (73%) ^b	16 (59%) ^d

^{a,b} Means without common superscripts differ P<0.05).

^{c,d} The percentage of ♀ calves from the sexed treatments (83%) differed from the control group, P<0.05, 1-tail, χ^2 .

Field trials 4, 5, and 6 (Tables 13, 14, 15) were done at the same location with 3 different groups of heifers. Unfortunately, it was not possible to replicate each trial similarly due to vagaries of field trials, such as scheduling personnel, availability of sexed semen from each bull, etc. The widely different pregnancy rates between trials 5 and 6 illustrate that conditions were different among trials. Some of the heifers in trial 6 were available because they failed to get pregnant after a month of natural mating. Under conditions of these trials, pregnancy rates were very similar between 1.5 and 3.0 x 10⁶ sexed, frozen sperm/dose. Furthermore, there was no advantage to uterine-horn insemination. There were no significant differences (P>0.05) in pregnancy rates among bulls except in Trial 5 in which the pregnancy rate of J2, 20/28 (71%), was higher than that of J4, 15/39 (38%) (P<0.05). This difference was not consistent from trial to trial, as J4 had numerically but not significantly (P>0.1) higher pregnancy rates than J2 in Trials 4 and 6.

Table 13. Results of field trial 4 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 33	No. pregnant day 64 to 67*
Sexed, frozen/body	1.5 x 10 ⁶	55	36 (65%) ^{a,b}	36 (65%) ^{a,b}
Sexed, frozen/body	3 x 10 ⁶	52	27 (52%) ^a	26 (50%) ^a
Control, frozen/body	20 x 10 ⁶	55	45 (82%) ^b	43 (78%) ^b

^{a,b} Means without common superscripts differ (P<0.01).

* Six heifers pregnant at d 30 to 33 were sold before the second pregnancy diagnosis; these were assumed to have remained pregnant.

Table 14. Results of field trial 5 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 35 ^a	No. pregnant day 60 to 62 ^a
Sexed, frozen/body	1.5 x 10 ⁶	23	12 (52%)	12 (52%)
Sexed, frozen/body	3.0 x 10 ⁶	25	15 (60%)	14 (56%)
Sexed, frozen/horns	1.5 x 10 ⁶	25	15 (60%)	12 (48%)
Sexed, frozen/horns	3.0 x 10 ⁶	25	17 (68%)	15 (60%)
Control, frozen/body	20 x 10 ⁶	30	20 (67%)	19 (63%)

^a No significant differences.

Table 15. Results of field trial 6 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 34	No. pregnant day 60 to 63
Sexed, frozen/body	1.5 x 10 ⁶	27	11 (41%) ^a	9 (33%) ^a
Sexed, frozen/body	3.0 x 10 ⁶	25	10 (40%) ^a	9 (36%) ^a
Sexed, frozen/horns	1.5 x 10 ⁶	24	8 (33%) ^a	7 (29%) ^a
Sexed, frozen/horns	3.0 x 10 ⁶	24	10 (42%) ^a	8 (33%) ^a
Control, frozen/body	20 x 10 ⁶	24	18 (75%) ^b	17 (71%) ^b

^{a,b} Means without common superscripts differ (P<0.05).

For trial 7 (Table 16), only one inseminator was available due to rescheduling. This is the only trial that showed a convincing advantage of uterine-horn over uterine-body insemination. For this inseminator under the conditions of the trial, 55% more heifers (22 percentage points) became pregnant with sexed, frozen semen inseminated into the uterine horns than into the uterine body. The true difference could be smaller because there are wide confidence intervals on these means. In all the other trials (5, 6, 9, and 11) in which body- and horn-insemination were compared, pregnancy rates were very similar for both methods for this technician as well as for other technicians.

Semen from one of the bulls used in Trial 7 was shipped without dilution from Montana by air in an insulated box at ~20°C before sorting; shipping time was 6 h. Pregnancy rates for the sexed sperm from the two bulls were virtually identical, 49% for the unshipped and 52% for the shipped semen. Semen was not diluted with extender and not cooled for shipping because staining properties of sperm with Hoechst 33342 are altered by dilution with extenders.

Furthermore, in other studies (see Example 4), storing semen neat at ambient temperature between collection and flow-sorting was found to be superior to diluting it.

Table 16. Results of field trial 7 -- Crossbred beef heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 37
Sexed, frozen/body	1.5×10^6	86	34 (40%) ^a
Sexed, frozen/horns	1.5×10^6	86	53 (62%) ^b
Control, frozen/body	20×10^6	35	18 (51%) ^{a,b}

^{a,b} Means without common superscripts differ ($P < 0.01$).

Field trial 8 (Table 17) concerned feedlot heifers not implanted with growth promotants; at the time pregnancy was diagnosed they were aborted, so calving data was not available. This experiment illustrates that efficacious sexing also can be done in the male direction. Pregnancy rates for the 2 bulls were 50 and 61%.

Table 17. Results of field trial 8 -- Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant ^a day 74 to 76	No. ♂ fetuses
Sexed, frozen, 72 mW laser/body	1×10^6	18	7 (39%)	6 (86%)
Sexed, frozen, 135 mW laser/body	1×10^6	18	13 (78%)	12 (92%)

^a No significant differences.

Field trial 9 (Table 18) was the only trial to show a convincing advantage of 3.0 versus 1.5 x 10⁶ sexed, frozen sperm/insemination dose. This advantage was true for both inseminators. Pregnancy rates for sexed sperm from the 2 bulls were 62 and 75%.

Table 18. Results of field trial 9 -- Red Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 60 to 63 ^a	No. ♀ fetuses
Sexed, frozen/body	1.5 x 10 ⁶	15	8 (53%)	7 (88%)
Sexed, frozen/body	3.0 x 10 ⁶	14	12 (86%)	9 (75%)
Sexed, frozen/horns	1.5 x 10 ⁶	16	9 (56%)	7 (78%)
Sexed, frozen/horns	3.0 x 10 ⁶	16	12 (75%)	11 (92%)
Control, frozen/body	20 x 10 ⁶	30	21 (70%)	13 (62%)

^a 3.0 x 10⁶ sexed sperm had a higher pregnancy rate (80%) than 1.5 x 10⁶ sexed sperm (55%), P<0.05, 1-tail χ^2 .

Pregnancy rates in field trial 10 (Table 19) with sexed, frozen semen, were similar to controls; the accuracy of sexing sperm on this trial was only 82%, which, however, is not significantly different from the targeted 90% accuracy. Pregnancy rates for sexed semen were 54, 66, and 50% for bulls AN4, AN7, and AN8, respectively (P>0.1). Eighteen of the heifers inseminated in this trial were the calves resulting from sexed sperm in field trial 1.

Table 19. Results of field trial 10 -- Angus heifers in Wyoming, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 61 to 63 ^a	No. ♀ fetuses
Sexed, frozen/body	1 x 10 ⁶	44	26 (59%)	23 (85%)
Sexed, frozen/body	3 x 10 ⁶	43	23 (53%)	17 (74%)
Control, frozen/body	20 x 10 ⁶	35	20 (57%)	12 (57%)

^a No significant differences.

Table 20. Results of field trial 11 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 28 to 30 ^a	No. pregnant day 56 to 58 ^{a,b}
5 Sexed, frozen/body	1 x 10 ⁶	12	8 (67%)	7 (58%)
Sexed, frozen/body	3 x 10 ⁶	12	6 (50%)	4 (33%)
Sexed, frozen/horns	1 x 10 ⁶	7	4 (57%)	4 (57%)
Sexed, frozen/horns	3 x 10 ⁶	7	4 (57%)	4 (57%)
10 Control, frozen/body	20 x 10 ⁶	9	4 (44%)	3 (33%)
^a No significant differences, χ^2 .				
^b 16 of 17 (94%) fetuses from the sexed semen treatments were female; 2 were too deep in the body cavity to sex with ultrasound.				

Data from trials were combined in which treatments were identical except 1 x 10 and 1.5 x 10⁶ sperm doses were pooled (Table 21).

Table 21. Meta-summary from combining trials with sexed, frozen semen and frozen controls.

Trials combined	Sperm no./site	No. heifers	No. pregnant
20 5, 6, 9, 11	1.0 - 1.5 x 10 ⁶ /body	77	36 (47%)
	3.0 x 10 ⁶ /body	76	38 (50%)
	1.0 - 1.5 x 10 ⁶ /horns	72	32 (44%)
	3.0 x 10 ⁶ /horns	72	39 (54%)
	20 x 10 ⁶ /body, control	93	61 (66%)
4, 5, 6, 9, 10, 11	1.0 - 1.5 x 10 ⁶ /body	176	98 (56%)
	3.0 x 10 ⁶ /body	171	88 (51%)
	20 x 10 ⁶ /body, control	183	124 (68%)
25 5, 6, 7, 9, 11	1.5 x 10 ⁶ /body	163	70 (43%)
	1.5 x 10 ⁶ /horn	158	85 (54%)
	20 x 10 ⁶ /body, control	128	79 (62%)

Pregnancy rates with sexed sperm were generally 70-90% of unsexed controls within experiments with 7 to 20 times more sperm. This difference was less in the more recent trials, possibly reflecting improved sexing and sperm-processing procedures.

In some trials, heifers were examined for pregnancy by ultrasound at both 1 and 2 months after insemination. Pregnancy losses in this interval were similar ($P>0.1$) for sexed (23/261; 8.8%) versus control (9/145; 6.2%) sperm treatments, which is one measure that genetic damage due to sexing is minimal. Calving information was obtained from only a few of the pregnant heifers because most cattle from the earlier trials were sold, and those from later trials have not calved yet. The population of calves produced to date from sexed semen appears to be no different from the population of controls.

CONCLUSION

Sex ratios in cattle can be distorted to about 90% of either sex by sorting sperm on the basis of DNA content with a flow cytometer/cell sorter followed by cryopreservation and relatively routine artificial insemination. Calves resulting from sexed sperm appear to be normal. For most bulls in these studies, pregnancy rates with 1.0 to 1.5×10^6 sexed, frozen sperm were 70 to 90% of unsexed controls with 20 or 40×10^6 frozen sperm inseminated conventionally. These results apply to well-managed heifers bred by well-trained inseminators using properly processed semen. There may be a small advantage to inseminating sexed sperm bilaterally into the uterine horns compared to standard uterine body insemination.

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the ends of the present invention.

All patents and publications described are herein incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

- 1 1. A method for the cryopreservation of sperm comprising:
 - 2 (a) obtaining a selected sperm sample;
 - 3 (b) cooling said selected sperm sample;
 - 4 (c) isolating sperm from said selected sperm sample to produce isolated sperm;
 - 5 (d) adding final extender to said isolated sperm to produce a suspension of
 - 6 sperm; and
 - 7 (e) freezing said suspension of sperm.
- 1 2. The method of Claim 1 wherein said selected sperm sample comprises a portion of the
2 sperm present in a source sample, said portion of sperm selected for a characteristic, and
3 wherein the sperm concentration in the selected sperm sample is lower than in the source
4 sample.
- 1 3. The method of Claim 1 wherein said selected sperm sample comprises sex-selected
2 sperm.
- 1 4. The method of Claim 1 wherein said selected sperm sample comprises mammalian
2 sperm.
- 1 5. The method of Claim 4 wherein said selected sperm sample comprises bovine sperm.
- 1 6. The method of Claim 4 wherein said selected sperm sample comprises equine sperm.
- 1 7. The method of Claim 4 wherein said selected sperm sample comprises porcine sperm.
- 1 8. The method of Claim 1 wherein said selected sperm sample comprises sperm selected by
2 a method from the group consisting of flow cytometry, a magnetic technique, a columnar
3 technique, a gravimetric technique, a biochemical technique, a technique based on

4 motility of sperm, a technique based on an electrical property of sperm, and any
5 combination thereof.

1 9. The method of Claim 8 wherein said sperm have been selected by flow cytometry.

1 10. The method of Claim 1 wherein cooling is carried out by reducing the temperature of the
2 selected sperm sample to about 5°Celsius.

1 11. The method of Claim 10 wherein cooling is carried out over a period of about 60 minutes
2 to about 240 minutes.

1 12. The method of Claim 1 wherein the final extender added to said selected sperm sample
2 each comprise, in addition to a cryoprotectant, one or more of the following components:
3 a component that maintains osmolality and buffers pH, an organic substance that reduces
4 cold shock and preserves fertility of sperm, an energy source, a substance that facilitates
5 sperm capacitation, and an antibiotic.

1 13. The method of Claim 12 wherein said cryoprotectant is selected from the group
2 consisting of disaccharides, trisaccharides, and any combination thereof.
3

1 14. The method of Claim 12 wherein said cryoprotectant is selected from the group
2 consisting of glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any
3 combination thereof.

1 15. The method of Claim 12 wherein said component that maintains osmolality and buffers
2 pH is selected from the group consisting of a buffer comprising a salt, a buffer containing
3 a carbohydrate, and any combination thereof.

1 16. The method of Claim 12 wherein said component that maintains osmolality and buffers
2 pH is selected from the group consisting of sodium citrate,
3 Tris[hydroxymethyl]aminomethane,

4 N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, monosodium glutamate,
5 milk, HEPES buffered medium, and any combination thereof.

1 17. The method of Claim 12 wherein said organic substance is selected from the group
2 consisting of egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin,
3 and any combination thereof.

1 18. The method of Claim 12 wherein said energy source is a monosaccharide selected from
2 the group consisting of glucose, fructose, mannose, and any combination thereof.

1 19. The method of Claim 12 wherein said antibiotic is selected from the group consisting of
2 tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin,
3 and any combination thereof.

1 20. The method of Claim 1 wherein, after the addition of the final extender, the sperm sample
2 and suspension of sperm, respectively, comprise glycerol, sodium citrate,
3 Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

1 21. The method of Claim 1 wherein, after the addition of the final extender, said sperm
2 sample and suspension of sperm, each comprise glycerol, sodium citrate, egg yolk, and
3 one or more antibiotics.

1 22. The method of Claim 1 wherein, after the addition of the final extender, said sperm
2 sample and suspension of sperm, each comprise glycerol, egg yolk, milk, fructose, and
3 one or more antibiotics.

1 23. The method of Claim 1 wherein said extender has a pH in the range of about 6.5 to about
2 7.5.

1 24. The method of Claim 1 wherein the sperm are isolated from said selected sperm sample
2 by centrifugation.

1 25. The method of Claim 24 wherein said centrifugation allows for at least about 50% to
2 about 90% recovery of sperm.

1 26. The method of Claim 1 wherein the concentration of sperm in said suspension prior to
2 freezing is about $1 \times 10^6/\text{ml}$ to about $300 \times 10^6/\text{ml}$.

1 27. A frozen selected sperm sample comprising a portion of the sperm present in a source
2 sample, said portion of sperm selected for a characteristic.

1 28. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 comprises sex-selected sperm.

1 29. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 comprises mammalian sperm.

1 30. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises bovine sperm.

1 31. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises equine sperm.

1 32. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises porcine sperm.

1 33. The frozen selected sperm sample of Claim 27 wherein the method used to select said
2 selected sperm sample comprises a technique from the group consisting of flow
3 cytometry, a magnetic technique, a columnar technique, a gravimetric technique, a
4 biochemical technique, a technique based on motility of sperm, a technique based on an
5 electrical property of sperm, and any combination thereof.

1 34. The frozen selected sperm sample of Claim 33 wherein said frozen selected sperm sample
2 comprises sperm that have been selected by flow cytometry.

1 35. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 is produced by a method comprising:

- 3 (a) obtaining a selected sperm sample;
4 (b) cooling said selected sperm sample;
5 (c) isolating sperm from said selected sperm sample to produce isolated sperm;
6 (d) adding final extender to said isolated sperm to produce a suspension of
7 sperm; and
8 (e) freezing said suspension of sperm.

1 36. A method comprising using the frozen selected sperm sample of Claim 27 for artificial
2 insemination or in vitro fertilization.

1 37. The method of Claim 36 comprising using said frozen selected sperm sample for low-
2 dose artificial insemination.

ABSTRACT OF THE INVENTION

The present invention provides a method of cryopreserving sperm that have been selected for a specific characteristic. In a preferred embodiment, the method is employed to freeze sex-selected sperm. Although the cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred. The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or bison sperm. The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

**DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION**

As a below named inventor,

I hereby declare that my residence, post office address and citizenship is as stated below next to my name.

I believe I am the original and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: "METHOD OF CRYOPRESERVING SELECTED SPERM CELLS", the specification of which

 X is attached hereto.

 was filed on and assigned
Application Serial Number

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign applications for patent or inventor's certificate, listed below and so identified, and I have also identified below any foreign application for patent or inventor's certificate on this invention filed by me or my legal representatives or assigns and having a filing date before that of the application on which priority is claimed.

Number	Country	Day/Month/ Year Filed	Priority Claimed - Yes or No

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Application Serial No.	Filing Date	Status

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States provisional application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior provisional application and the filing date of this application.

Provisional Application No.	Filing Date	Status
60/167,423	November 24, 1999	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorneys and agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith and request that all correspondence and telephone calls with respect to this application be directed to McCUTCHEN, DOYLE, BROWN & ENERSEN, LLP Three Embarcadero Center, San Francisco, California 94111, Telephone No. (415) 393-2000:

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FEE DETERMINATION	PA	70385	2/2
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INDEX OF CLAIMS

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Claim	Final	Original	Date
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SEARCHED			
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INTERFERENCE SEARCHED			
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SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
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WEST	12/15/00	MM/M
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UTILITY PATENT APPLICATION TRANSMITTAL (Only for new nonprovisional applications under 37 CFR 1.53(b))		Attorney Docket No. 22091-701	Total Pages
First Named Inventor or Application Identifier Schenk, John L.			
Express Mail Label No. EL29073605TUS			

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents:

1. ☒ Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)
(Total Pages)
2. ☒ Specification
(preferred arrangement set forth below)
 - Descriptive title of the Invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure(Total Sheets)
(Total Pages)
3. ☐ Drawings (35 USC 113)
4. Oath or Declaration
 - a. ☒ New
 - b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]
 - i. ☐ **DELETION OF INVENTOR(S)**
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b) (useable if Box 4b is checked) The entire disclosure of the
5. ☐ Incorporated By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program (Appendix)
7. ☐ Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
 - a. ☐ Computer Readable Copy
 - b. ☐ Paper Copy (identical to computer copy)
 - c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☒ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement (when there is an assignee) ☒ Power of Attorney (in Declaration)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS/PTO-1449) ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503) (Should be specifically itemized)
14. ☒ Small Entity Statement(s) ☐ Statement filed in prior application, Status still proper and desired
15. ☐ Certified Copy of Priority Document (s) (if foreign priority is claimed)
16. ☐ Other:

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| ☐ Continuation | ☐ Divisional | ☐ Continuation-in-part (CIP) |
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- of prior application No: _____

18. CORRESPONDENCE ADDRESS

☐ Customer Number or Bar Code Label	(Insert Customer No. or Attach Bar code label here)	or ☒ Correspondence address below

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TOTAL AMOUNT OF PAYMENT (\$573.00)

Complete if Known

Application Number	To be assigned
Filing Date	Herewith
First Named Inventor	Schenk, John L.
Examiner Name	To be assigned
Group / Art Unit	To be assigned
Attorney Docket No.	22091-701

METHOD OF PAYMENT (check one)

1. ☐ The Commissioner is hereby authorized to charge the indicated required fees to:
- ☐ The Commissioner is hereby authorized to charge any additional required fees and credit any over payments to:

Deposit Account Number: 13-0257
Deposit Account Name: McCutchen, Doyle, Brown & Enersen LLP

2. ☒ Payment Enclosed:
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FEE CALCULATION

1. BASIC FILING FEE

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description	Fee Paid
101	760	201	380	Utility filing fee	380.00
106	310	206	155	Design filing fee	
107	480	207	240	Plant filing fee	
108	760	208	380	Reissue filing fee	
114	150	214	75	Provisional filing fee	

SUBTOTAL (1) (\$) 380.00

2. EXTRA CLAIM FEES

	Extra Claims	Fee from below	Fee Paid
Total Claims	37	20** = 17	9 = 153.00
Independent Claims	3	3** = 0	39 = 0
Multiple Dependent			

**or number previously paid, if greater; For Reissues, see below

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description
103	18	203	9	Claims in excess of 20
102	78	202	39	Independent claims in excess of 3
104	260	204	130	Multiple dependent claim, if not paid
109	78	209	39	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) (\$) 153.00

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Fee Code	Entity Fee (\$)	Small Fee Code	Entity Fee (\$)	Fee Description	Fee Paid
105	2,130*	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet.	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	380	216	190	Extension for reply within second month	
117	870	217	435	Extension for reply within third month	
118	1,360	218	680	Extension for reply within fourth month	
128	1,850	228	925	Extension for reply within fifth month	
119	300	219	150	Notice of Appeal	
120	300	220	150	Filing a brief in support of an appeal	
121	260	221	130	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,210	241	605	Petition to revive - unintentional	
142	1,210	242	605	Utility Issue fee (or reissue)	
143	430	243	215	Design Issue fee	
144	580	244	290	Plant Issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt	40.00
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	760	246	380	Fill a submission after final rejection (37 CFR 1.129(a))	
149	760	249	380	For each additional invention to be examined (37 CFR 1.129(b))	

Other fee (specify) _____
Other fee (specify) _____

* Reduced by Basic Filing Fee Paid

SUBTOTAL (3) (\$) 40.00

SUBMITTED BY

Typed or Printed Name: Emily M. Haliday
Signature: *Emily M. Haliday*

Date: Jan 5, 2000

Complete (if applicable)

Reg. Number: 38,903
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METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 The applications claims the benefit of U.S. Provisional Application No. 60/167,423, filed November 24, 1999.

FIELD OF THE INVENTION

The invention relates to a method for freezing sperm selected for a particular characteristic, as well as to a frozen selected sperm sample and methods of using such a sample.
10 The invention is particularly useful for preserving sex-selected sperm.

BACKGROUND OF THE INVENTION

Over half a century ago, artificial insemination was introduced in the United States as a commercial breeding tool for a variety of mammalian species. Although artificial insemination was initially limited to regions relatively close to the site of sperm collection, advances in the cryopreservation and storage of sperm have facilitated widespread distribution and commercialization of sperm intended for artificial insemination or in vitro fertilization.
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Further improvements in mammalian sperm collection, selection, cryopreservation, storage, and handling techniques have enhanced the ability of breeders to produce animals having desired traits. For example, advances in selection of mammalian sperm based on slight differences in physical characteristics has made it possible to separate sperm based on sex-type, that is, to select for cells containing either the X or Y chromosome. This technique allows the breeder to manipulate the relative percentage of X- or Y-type sperm in a sample and thereby determine offspring sex. The ability to select sperm based on sex-type or any other desirable characteristic provides an important tool for accelerating genetic progress, increasing production efficiency, and achieving greater flexibility in livestock management. Full exploitation of this tool, however, depends on the ability to freeze and store selected sperm.
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A variety of methods are available for selecting cells; however, the selection and subsequent processing of sperm presents unique challenges because sperm are incapable of DNA repair and because of sperm morphology. Each sperm has an acrosome overlying the head and a tail, which are important for fertility and which are relatively susceptible to physical injury. In
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addition, sperm fertility decreases with increasing time between collection and use. As most of the available selection methods involve physical stresses and take time, selected sperm are typically somewhat compromised compared to non-selected cells. Fertility may be further reduced if the selection technique involves significant dilution. It has been suggested that this

5 "dilution effect" may be due to the loss of protective components in seminal plasma.

Flow cytometry is a particularly efficient selection method that has been employed for sorting sperm by sex-type. However, sorted sperm are subject to stresses beyond those normally encountered in standard artificial insemination or in vitro fertilization protocols. In particular, flow cytometry is time consuming, and, because of the physical constraints of flow cytometers,

10 sperm must be diluted for sorting to levels that are not optimal for storage (usually to on the order of 10^5 - 10^6 /ml). Furthermore, sorted sperm intended for artificial insemination must be concentrated so that conventional packaging and delivery equipment can be used. The need for a concentration step thus exposes already somewhat compromised sperm to additional physical stresses.

15 The freezing of sperm also invariably reduces fertility, motility, and/or viability, and, although techniques for freezing unselected sperm are well known, no technique for cryopreservation of selected sperm has been described.

SUMMARY OF THE INVENTION

20 The present invention provides a method of cryopreserving sperm that have been selected for a specific characteristic. The method is particularly useful for cryopreserving sperm selected by a method that results in dilution of the sperm, since the method provides for the isolation of sperm from a selected sperm sample, followed by addition of a final extender to the isolated sperm to produce a suspension having a desired concentration of sperm. In a preferred

25 embodiment, the method is employed to freeze sex-selected sperm. Although the cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred.

The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample

30 includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or

bison sperm. Also within the scope of the invention is a container including a frozen sperm sample according to the invention.

The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

DETAILED DESCRIPTION OF THE INVENTION

The present invention allows cryopreservation of sperm that have been selected for a particular characteristic, facilitating storage and/or shipment of selected sperm samples to sites distant from the collection site. Thawing yields viable sperm that can be used in procedures such as artificial insemination ("AI") and in vitro fertilization ("IVF"). This result was surprising because of the well-documented fragility of sperm. Prior researchers had demonstrated that the stresses associated with various selection methods or with cryopreservation resulted in significant losses in fertility and/or viability. The present inventors have demonstrated, for the first time, that pregnancies can be achieved with sperm that have been selected and then frozen.

The invention represents an important advance in livestock management, where selection of sperm for use in such procedures can be used to increase the production of offspring having desirable traits. For example, selection to obtain sperm carrying either the X or the Y chromosome allows control over offspring sex, which is advantageous for producers of animals such as dairy or beef cattle. Sex selection also finds application in breeding valuable (e.g., show or race horses) or endangered animals. The ability to freeze selected sperm, which the invention provides, will enable widespread use of such selection methods to, e.g., increase livestock production efficiency as well as quality.

Definitions

The term "acrosome" or "acrosomal cap" refers to the cap that covers the anterior half of the head of sperm and that contains enzymes necessary for ovum penetration.

The term "sex-type" refers to the type of sex chromosome present in the sperm (i.e., the X or Y chromosome).

The term "capacitation" refers to the specific changes a sperm undergoes to develop the capacity to fertilize ova, such as enzymic changes on the surface of the acrosome that lead to release of acrosomal enzymes that facilitate penetration of the sperm into the ovum.

As used with reference to sperm, the term "cryoprotectant" refers to a molecule that protects sperm during a freeze-thaw cycle, promoting survival and retention of fertilizing capacity.

The term "dilution effect" refers to the rapid decline in motility and/or viability of sperm when highly diluted.

As used herein, the term "selection" refers to a method whereby a sample is subdivided based on presence or absence of a specific characteristic (unless context dictates otherwise). Thus, a "selected sperm sample" is a sample obtained by subjecting a source sample to selection for the specific characteristic. A selected sperm sample is therefore enriched, relative to the source sample, in sperm having the specific characteristic.

The term "sorting" is used herein to describe a selection method carried out using a fluorescence-activated cell sorter (FACS).

The term "extender" refers to any medium that tends to preserve sperm viability. The term "extension" refers to the dilution of sperm with extender.

The term "initial extender" refers to a medium used to extend sperm prior to the isolation step of the method of this invention.

The term "final extender" refers to a medium used to extend sperm prior to the freezing step of the method of this invention.

An "organic substance" in an extender described herein is any organic substance that tends to reduce cold shock and preserve fertility of sperm.

An "energy source" in an extender described herein is any substance or substrate that sperm can utilize for cell maintenance and/or motility.

The term "osmolality," as used herein, is a measure of the osmotic pressure of dissolved solute particles in an aqueous solution (e.g., an extender). The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of water.

Cryopreservation Method

The invention provides a method of cryopreserving selected sperm includes the following

steps:

- (1) obtaining a selected sperm sample;
- (2) cooling the selected sperm sample;
- (3) isolating sperm from the selected sperm sample;
- (4) adding final extender to the isolated sperm to produce a suspension of sperm; and
- (5) freezing the suspension of sperm.

10 *Obtaining a Selected Sperm Sample*

The first step in the cryopreservation method of the invention encompasses obtaining a previously selected sperm sample, as well as subjecting a source sample to any suitable selection method. Sperm from any species can be selected and frozen according to the method of the invention. The method can be carried out with sperm from domesticated animals, especially livestock, as well as with sperm from wild animals (e.g., endangered species). Preferably, the selected sperm sample contains mammalian sperm. Human sperm, bovine, equine, porcine, ovine, elk, and bison sperm are particularly preferred.

Generally, the selected sperm sample contains normal, viable sperm. To this end, the ejaculate from which the sperm are obtained typically has at least about 50%, and preferably at least about 75% morphologically normal sperm. In these embodiments, generally at least about 40%, and preferably at least about 60% of the sperm in the ejaculate exhibit progressive motility.

A wide variety of methods for selecting cells from a mixed populations are available, including, for example, selection based on binding of cells or cell components with antibodies, antibody fragments, or other binding partners and differential staining.

The invention is exemplified herein with selection based on sex-type, and sex-selected sperm for use in the invention can be obtained using any selection strategy that takes advantage of slight differences in characteristics between X- and Y-type sperm. Exemplary sex-selection methods include magnetic techniques (see, e.g., U.S. Patent No. 4,276,139), columnar techniques (see, e.g., U.S. Patent No. 5,514,537) gravimetric techniques (see, e.g., U.S. Patent No. 3,894,529, reissue Patent No. 32350, U.S. Patent Nos. 4,092,229, 4,067,965, and 4,155,831). Sex-selection based on differences in electrical properties is disclosed in U.S. Patent

No. 4,083,957, and techniques that select based on differences in electrical and gravimetric properties are discussed in U.S. Patent Nos. 4,225,405, 4,698,142, and 4,749,458. U.S. Patent Nos. 4,009,260 and 4,339,434 describe selection based on differences in motility. Biochemical techniques relying on antibodies are disclosed in U.S. Patent Nos. 4,511,661, 4,999,283, 3,687,803, 4,191,749, 4,448,767, whereas U.S. Patent Nos. 5,021,244, 5,346,990, 5,439,362, and 5,660,997 describe selection based on differences in membrane proteins.

Flow cytometry is a preferred method for separating cells from mixed populations based on differential staining with fluorescent dyes or binding to fluorescently labeled molecules, such as antibodies or nucleic acids. In fluorescence activated cell sorting ("FACS"), cells are "sorted" into different populations based on the fluorescence intensity upon irradiation. FACS can be used for sex-selection of sperm because the X chromosome contains slightly more DNA than the Y chromosome. When sperm are stained with a fluorescent DNA-binding dye, X-chromosome bearing sperm absorb more dye than Y-chromosome bearing sperm and the two populations can therefore be separated by FACS. This strategy was discussed in U.S. Patent No. 4,362,246 and significantly expanded upon in U.S. Patent No. 5,135,759 (issued to Johnson). Separation has been enhanced through the use of high-speed flow cytometers, such as the MoFlo® flow cytometer produced by Cytomation, Inc. (Ft. Collins, CO) and described in U.S. Patent Nos. 5,150,313, 5,602,039, 5,602,349, and 5,643,796, as well as in PCT Publication No. WO 96/12171.

The selection method used to obtain the selected sperm sample is preferably one that preserves sperm viability. Because of the relative fragility of sperm, normal flow cytometry techniques should generally be modified for sorting sperm. More specifically, the flow cytometry entails staining, dilution, and interrogation of cells with light. All of these steps represent stresses that can reduce sperm viability. The sensitivity of sperm to these stresses can vary between species and even between individuals within species. Such sensitivities have either been documented or can readily be determined by empirical studies, such as those described in Examples 1-5.

Modifications that enhance viability are described the patent publications discussed above. For instance, procedures that provide improved sheath and collector systems for sorting sperm are disclosed in PCT Publication No. WO 99/33956 (Application No. PCT/US98/27909). Further, Examples 1-7 below describe exemplary procedures for staining and sorting sperm.

Example 3 describes a study of the effects of laser intensity and dye concentration of post-thaw motility of sorted frozen sperm. This study indicates that the use of lower laser intensities during sorting can increase post-thaw motility.

5 The selected sperm sample can contain a variety of components besides sperm and will often contain components added to protect the sperm during the selection process. In the case of FACS, the selected sperm sample can contain component(s) of the solutions used for staining and sorting (e.g., the sheath fluid and the catch buffer).

10 In addition, the selected sperm sample typically contains an extender or extender fraction. For example, "two-step" extenders including an "A fraction" lacking glycerol and a "B fraction" containing glycerol are well known. The A fraction is added to sperm first, followed by addition of an equal volume of the B fraction. For this step, the B fraction is often divided into at least two aliquots and added sequentially; e.g., the second B fraction aliquot is added 15 minutes after the first.

15 If no extender components are present, an extender or extender fraction is typically added to the selected sperm sample before the sperm are isolated from the sample. If only some extender components are present, additional components can optionally be added so that selected sperm sample includes a complete extender or an extender fraction before the isolation step. In exemplary embodiments, bovine sperm are flow-sorted so as to produce a selected sperm sample including the A fraction of an extender (see Examples 2, 3, and 4). If desired, the B fraction can then be added to the selected sperm sample before the isolation step (see Example 5). The pre-isolation step extender (or extender fraction) is termed "the initial extender" to distinguish it from the "final extender" employed for the extension of isolated sperm before freezing. If the selected sperm sample was selected using FACS, the initial extender can be matched to the sheath fluid employed for sorting. Exemplary matched sheath fluids and extenders are described in detail in Example 4.

25 An extender suitable for use in the selected sperm sample includes a physiologically acceptable carrier. The physiologically acceptable carrier is typically aqueous, and, in preferred embodiments, includes deionized water. Suitable extenders commonly comprise one or more of the following additional components: a cryoprotectant, a component that maintains osmolality and buffers pH, an organic substance that prevents cold shock and preserves fertility of sperm, a detergent that acts synergistically with certain organic substances to enhance preservation of

sperm, an energy source that can be readily utilized by sperm, an antioxidant, which protects sperm from cold shock, a substance that facilitates sperm capacitation, and one or more antibiotics.

Although cryoprotectants useful in the invention are not limited to those acting by a particular mechanism, most conventional cryoprotectants act, at least in part, by reducing intracellular dehydration. Specifically, freezing is accompanied by an increase in solute concentration in the medium surrounding sperm. This increase in solute concentration draws water out of the cells, which increases intracellular electrolyte concentration. Exemplary cryoprotectants include glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and the like. The cryoprotectant suitable for use in a given extender can vary, depending on the species from which sperm are derived. For example, glycerol is suitable for use in cryopreservation of human and bovine sperm, but is generally not used for cryopreservation of porcine or rabbit sperm. Such preferences are well known for many commercially valuable sperm and can readily be determined empirically for other types of sperm.

The extender useful in the invention optionally includes one or more components that help maintain osmolality and provide buffering capacity. In preferred embodiments of the invention, the osmolality of the extender approximates that of physiological fluids. More preferably, the osmolality of the extender is in the range of about 280 mOsm to about 320 mOsm. The pH is also preferably within a physiologically acceptable range, more preferably in the range of about 6.5 to about 7.5.

Substances helpful in maintaining osmolality and pH within these ranges are well known in the art and can be added to the extender as a solid or already in solution. A buffer containing a salt, a carbohydrate, or a combination thereof can be employed for this purpose. Specific examples include sodium citrate, Tris[hydroxymethyl]aminomethane, and TES (N-Tris [Hydroxymethyl]methyl-2-aminoethanesulfonic acid), and monosodium glutamate buffers; milk; HEPES-buffered medium; and any combination thereof. The component employed to help maintain osmolality and provide buffering capacity in a particular application can vary depending on the other components of the extender and, in some cases, on the species from which the sperm are derived. The selection of such a component for use in the present invention is, however, within the level of skill in the art.

One or more organic substances that protect sperm against cold shock and help preserve fertilizing capacity can also be included in the extender. Such substances are well known and are sometimes described as "nonpenetrating cryoprotectants." One skilled in the art can readily determine an organic substance suitable for a particular application of the cryopreservation method described herein. For example, organic substances containing protective constituents (e.g., lipoproteins, phospholipids, lecithin) that are believed to reduce the impact of cold shock and the dilution effect can be included in the extender. Suitable organic substances include disaccharides, trisaccharides, and any combination thereof. Exemplary organic substances include egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, cholesterol, and any combination thereof.

The extender can also include a detergent. Alkyl ionic detergents, such as sodium dodecyl sulfate (SDS), have been reported to act synergistically with egg yolk to enhance protection against cold shock. Other detergents useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular detergent for a specific application is within the level of skill in the art in light of the guidance provided herein. See, e.g., Example 5.

Preferably, the extender includes an energy source that is readily utilized by sperm. In the absence of an energy source, sperm may oxidize intracellular phospholipids and other cellular components. Thus, the inclusion of an energy source in the extender protects intracellular reserves and cellular components. As is well known in the art, sugars, particularly monosaccharides, provide a convenient energy source, although any conventional energy source can be employed in the extender. Exemplary monosaccharides useful in the extender include glucose, fructose, and/or mannose.

One or more antioxidants can optionally be included in the extender to provide additional protection against cold shock. Exemplary antioxidants include butylated hydroxytoluene (BHT), its derivatives, and the like. However, other antioxidants useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular antioxidant for a specific application is within the level of skill in the art in light of the guidance provided herein.

The extender can also contain a substance that facilitates sperm capacitation. A variety of capacitation facilitators are known in the art and any can be employed in the extender. Examples

include enzymes such as alpha amylase, beta amylase, beta glucuronidase, which can be used in combination, if desired.

Finally, the extender preferably includes an antibiotic, since substantial bacterial growth can threaten sperm viability and increase the risk of infection of the host in artificial insemination or in vitro fertilization procedures. Any of a variety of antibiotics useful in the cryopreservation of cells can also be employed in the extender. The selection of a suitable antibiotic depends on the species from which the sperm was obtained, the procedures involved in obtaining and handling the sperm sample, and the specific microorganism(s) to be targeted. Exemplary antibiotics include tylosin, gentamicin, lincomycin, spectinomycin, linco-spectin (a combination of lincomycin and spectinomycin), penicillin, streptomycin, and ticarcillin, which can be used alone or in combination. However, one skilled in the art can readily determine other antibiotics suitable for use in the extender.

Exemplary extenders are discussed in greater detail below and in the examples.

The sperm concentration is typically lower in the selected sperm sample than in the source sample, and, as indicated above, when FACS is employed, the dilution is significant. A typical sort based on sex-type can produce a sample containing sperm at 6×10^5 cells/ml catch buffer. As such a low concentration is not optimal for storage (at least for most species tested), the cryopreservation method of the invention generally concentrates the selected sperm sample.

Cooling the Selected Sperm Sample

The second step in the cryopreservation method entails cooling the selected sperm sample, typically, by reducing the temperature at a controlled rate. Cooling too rapidly can cause cold shock, which can result in a loss of membrane integrity and cell function. The severity of the effects of cold shock vary from species to species and depend on factors such as the rate of cooling and the temperature range. Under suitable controlled cooling conditions, the sperm are able to adapt to thermal effects. Example 2, among others, describes conditions for cooling bovine sperm, and determining suitable conditions for cooling sperm of other species is within the level of skill in the art.

In a preferred embodiment of the invention, the selected sperm sample is cooled typically from about 22°Celsius, to about 5°Celsius, and cooling is generally carried out over a period of about 60 minutes to about 24 hours, preferably over a period of about 90 minutes to about 240

minutes, and most preferably over a period of about 90 minutes to about 120 minutes. Cooling can be accomplished by any convenient method, including simply placing the selected sperm sample in a 5°Celsius environment.

5 *Isolation of Sperm Cells from the Selected Sperm Sample*

After initial extension of the selected sperm sample, sperm are isolated from the sample using any sufficiently gentle isolation method that provides at least about 50% recovery of sperm, more preferably about 75% to about 90% recovery of sperm, and most preferably about 80% to about 90% recovery of sperm. During the isolation step, the cooled sperm should
10 generally be kept cold, i.e., between about 1 and about 8°Celsius, and preferably close to 4 or 5°Celsius.

Any of a variety of methods suitable for recovering cells from a suspension can be used to isolate the sperm, including for example, filtration, sedimentation, and centrifugation. In an exemplary, preferred embodiment, the selected sperm sample is aliquoted into 50 ml tubes at
15 volumes not exceeding about 27 ml, and preferably between about 20 to about 27 ml. Centrifugation is carried out at about 4°Celsius, at about 850 x g, for about 20 minutes. Preferably, the centrifugation step provides at least about 50% to about 90% recovery of sperm, more preferably about 60% to about 90% recovery of sperm, and most preferably about 70% to about 90% recovery of sperm. After isolation, the supernatant is removed and the pellet is
20 suspended by vortexing gently or repeated aspiration at 4°Celsius. The sperm concentration is then typically determined (e.g., using a hemacytometer).

Final Extension of Isolated Sperm Cells

After isolation, the sperm are pooled, if desired, and extended with final extender to an
25 appropriate concentration for freezing. The concentration of sperm after the final extension and prior to freezing is preferably in the range of about 1×10^6 /ml to about 300×10^6 /ml, more preferably about 10×10^6 /ml to about 50×10^6 /ml, and most preferably about 10×10^6 /ml to about 20×10^6 /ml.

The description of the initial extender above also applies to the final extender, which can
30 be the same as or different from the initial extender. In particular embodiments, the composition



of the sperm sample extended with the final extender is substantially similar to (if not the same as) the composition of the sperm sample after addition of the initial extender.

In a preferred embodiment of the invention, an egg yolk-Tris extender is used. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); citric acid
5 and Tris[hydroxymethyl]aminomethane (buffer); egg yolk (organic substance); fructose (energy source); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

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Components of Egg Yolk-Tris Extender

Glycerol:	4-8% vol/vol
Citric Acid:	55-75 mM
Tris [hydroxymethyl]aminomethane:	190-210 mM
Egg yolk:	5-25% vol/vol
Fructose:	45-65 mM
Tylosin:	25-100 µg/ml
Gentamicin:	200-300 µg/ml
Linco-spectin:	100-400 µg/ml*

* 100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin

In a variation of this embodiment particularly suitable for freezing bovine sperm, the concentrations of these components after addition of the final extender to the isolated sperm are about 6% (vol/vol) glycerol, about 65 mM citric acid, about 200 mM

Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 56 mM fructose, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 150/300 µg/ml linco-spectin (i.e., 150 µg/ml lincomycin and 300 µg/ml spectinomycin), in deionized water.

In an alternative embodiment, an egg yolk-citrate extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); sodium citrate (buffer); egg yolk (organic substance); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

Components of Egg Yolk-Citrate Extender

Glycerol:	4-8% vol/vol
Sodium Citrate :	60-80 mM
Egg yolk:	5-25% vol/vol
Tylosin:	25-100 µg/ml
Gentamicin:	200-300 µg/ml
Linco-spectin:	100-400 µg/mL*

* 100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin

Exemplary, preferred concentrations for freezing bovine sperm are about 7% (vol/vol) glycerol, about 72 mM sodium citrate, about 20% (vol/vol) egg yolk, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

In another alternative embodiment, an egg yolk-TES-Tris ("EY TEST") extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); egg yolk and heated milk, e.g., homogenized milk containing 1.25% fructose with 10% glycerol (organic substances); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

Components of Egg Yolk TES-Tris Extender

	Glycerol:	3-7% vol/vol
	Tris [hydroxymethy-methyl]-2-aminoethanesulfonic acid:	140-170 mM
5	Tris [hydroxymethyl]aminomethane:	60-80 mM
	Egg yolk:	5-25% vol/vol
	Fructose:	5-12 mM
	Tylosin:	50-150 µg/ml
	Gentamicin:	400-600 µg/ml
10	Linco-spectin:	200-700 µg/mL*
	*200-700 µg/ml lincomycin and 200-700 µg/ml spectinomycin	

Exemplary, preferred concentrations for freezing bovine sperm are about 5% (vol/vol) glycerol, about 158mM Tris[hydroxymethy-methyl]-2-aminoethanesulfonic acid, about 72 mM
15 Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 8mM fructose, about 100 µg/ml tylosin, about 500 µg/ml gentamicin, and about 300/600 µg/ml linco-spectin.

In yet another alternative embodiment of the invention, a Milk extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); heated homogenized milk (organic substance); fructose (energy source); and tylosin, gentamicin,
20 and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

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Components of Milk Extender

	Homogenized Milk	90% (vol/vol)
	Glycerol:	3-7% (vol/vol)
5	Fructose:	1.25 % (wt/vol)
	Tylosin:	50 µg/ml
	Gentamicin:	250 µg/ml
	Linco-spectin:	250/300 µg/ml

*
10 * 250-300 µg/ml lincomycin and 250-300 µg/ml spectinomycin

Exemplary preferred concentrations for freezing bovine sperm are about 90% milk, about 10% (vol/vol) glycerol, about 1.25% fructose (wt/vol?), about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

15 Other extenders standardly used to freeze sperm can also be employed as the final extender in freezing selected sperm. A variety of extenders optimized for use in freezing sperm from various species have been described, and many are commercially available. Freezing extenders for equine sperm typically consist of milk, egg yolk, various sugars, electrolytes and a cryoprotectant. Exemplary freezing extenders are described by Squires, E.L., et al., Cooled and
20 Frozen Stallion Semen Animal Reprod. and Biotechnology Laboratory, Bulletin No. 69, Chapter 8, "Seminal Extenders" pp. 49-51 (July, 1999).

Equilibration and Freezing of Sperm

25 Extension of the sperm sample produces a suspension of sperm, which is then transferred into containers for freezing. If the sperm are intended for use in fertilization, the cells are conveniently aliquoted into individual doses sufficient to achieve fertilization. The required dose can vary substantially from one species to the next and is either well-known (e.g., for cattle and horses) or can readily be determined. In the case of sex-selected bovine sperm, convenient doses range from about 1.0×10^6 sperm to about 3.0×10^6 sperm.

30 Any suitable container can be employed for freezing, including, for example, an ampule, a vial, and a straw. Sperm intended for AI are typically frozen in straws (e.g., 0.25 ml or 0.50 ml

straws) designed for use with an insemination gun. Preferably, a bolus of extender is drawn into the straw, followed, in sequence, by air, sperm, air, and extender, so that the sperm are flanked on either side by an air space, which separates the sperm from a bolus of extender at either end of the straw.

5 Prior to freezing, the sperm are generally allowed to equilibrate at about 5°C. Preferably, the sperm are allowed to equilibrate for a period in the range of about 1 hour to about 18 hours, more preferably between about 3 hours and about 18 hours, and most preferably between about 3 hours and about 6 hours (see Example 2).

10 Following equilibration, any standard freezing method can be employed, provided the freezing rate is not too rapid (i.e., not in excess of about 0.5°C/minute). Preferably, the freezing rate is about 0.5°C/minute. In an exemplary, preferred embodiment, the sperm are placed in static liquid nitrogen vapor, and freezing is carried out in three distinct stages over a period of about 10 minutes. In the first stage of freezing, the sperm are cooled from about 5°C to about -15°C at a rate of about 40°C/minute to about 65°C/minute. In the second stage of freezing, the sperm are cooled from about -15°C to about -60°C at a rate of about 25°C/minute to about 35°C/minute. In the third stage, the sperm are plunged into liquid nitrogen at about -100°C.

Selected Sperm Samples

20 In addition to a freezing method, the invention provides a frozen sperm sample including sperm selected from a source sample for a particular characteristic. The sperm can be from any species, including any of those discussed above with reference to the freezing method. The invention encompasses frozen sperm selected for any characteristic by any suitable method, such as those described above. Preferred embodiments include frozen sex-selected human, bovine, equine, porcine, ovine, elk, or bison sperm. Sex-selection is preferably carried out using flow cytometry as described generally above.

25 Also within the scope of the invention is a container containing a frozen sperm sample according to the invention. The container can be formed from any material that does not react with the frozen sperm sample and can have any shape or other feature that facilitates use of the sample for the intended application. For samples intended for use in AI, for example, the container is conveniently a straw (e.g., 0.25 ml or 0.5 ml straw) designed for use with an insemination gun. The container is sealed in any manner suitable for preserving the sample at

the intended storage temperature, which is typically below -80°Celsius. 0.25 ml straws can be sealed, for instance, with PVC powder, ultrasonically, or with a cotton-polyvinyl plug and/or a stainless steel ball (BB).

5 As the frozen sperm sample of the invention is typically thawed before use, the invention also provides a thawed, previously frozen, selected sperm sample and a container including such a thawed sample.

Methods of Using the Selected Sperm Sample

10 The frozen selected sperm sample of the invention is suitable for use in any method in which sperm are used. The sample can be thawed and used in any conventional fertilization method, such as artificial insemination or in vitro fertilization. Thawing is carried out in the same manner as for frozen, non-selected sperm. Briefly, the straw containing the frozen sperm is submerged in a water bath maintained at a temperature of about 35°C to about 37°C for a period of about 20 to about 30 seconds. After thawing, semen deposition (e.g., insemination) is carried out according to standard procedures, taking care to protect the sperm from environmental
15 fluctuations.

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EXAMPLES

Example 1

Effects Of Dilution On Sperm

- 5 Objective: to determine the effect of sperm concentration on sperm motility for non-frozen, non-sorted, but highly diluted sperm.

A. *Effects of Dilution on Non-washed Sperm*

1. Collection of Source Sample. Sperm were collected from bulls on a routine
10 collection schedule using an artificial vagina as described in Schenk J., Proc 17th NAAB, p.48-58 (1998), and Saacke RG, Proc NAAB Tech Conf AI Reprod. 41:22-27 (1972). All ejaculates used contained greater than 50% progressively motile and greater than 75% morphologically normal sperm. Antibiotics were added to the raw ejaculate as described by Shin S., Proc NAAB Tech Conf AI Reprod. 11:33-38 (1986) within 15 minutes of collection, and the concentration of
15 sperm was determined using a spectrophotometer.

2. Methods. Sperm from 4 bulls were diluted to 1.25, 2.5, 5, 10, 15, and 20×10^6 /ml using an egg yolk-citrate extender (EYC) prepared with 20% egg yolk (vol/vol) in 72 mM sodium citrate, 50 μ g/ml tylosin, 250 μ g/ml gentamicin, and 250/300 μ g/ml linco-spectin. Each
20 sample was prepared in duplicate (2 tubes/dilution/bull) and comprised 8 ml total volume per tube. All samples were incubated for 60 minutes at 22°C, after which they were centrifuged using a swinging bucket centrifuge (Eppendorf, Model # 5810R) at $600 \times g$ for 10 minutes to concentrate the sperm. After centrifugation, the supernatant from one set of the duplicate tubes was not removed; the sperm were resuspended in the same medium and at the original
25 concentration by repeated gentle aspiration using a 5-ml serological pipette. (The second set of the duplicate tubes were used in Example 1B.) Sperm samples were then cooled to 5°C at 0.2°C/min over 90 minutes. These sperm were termed "non-washed sperm." All samples were incubated at 5°C for 24 or 48 h post-collection.

3. Evaluation of Motility. After incubation, the samples were warmed to 37°C using
30 a dry block incubator for 10 minutes prior to determination of motility. For this experiment, a

single, blind estimate of the percentage of progressively motile sperm was determined for each sample. Progressive sperm motility was determined subjectively for each subclass by a single observer (x200, phase-contrast microscopy); another person prepared the microscope slides in a randomized manner so the observer was unaware of treatments.

5

4. Statistical Analysis. Data were analyzed by analysis of variance (SAS Institute, Cary, North Carolina) with factors bulls and initial dilution concentration. Separate analyses were done for each incubation time. Dilution trends were tested using (log) linear contrasts.

5. Results. Data for non-washed sperm (Table 1) revealed (log) linear relationships (P<0.01) for both incubation times. Percentages of motile sperm increased as sperm concentration increased from $1.25 \times 10^6/\text{ml}$ to $10 \times 10^6/\text{ml}$, but there was little difference thereafter. The cubic term was significant (P<0.05) for 24-h and marginally significant (P<0.1) for 48-h incubations. There was a bull effect (P<0.01) at both times.

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Table 1. Effects of cooling on non-washed sperm motility (%) after cooling to 5°C.

Dilution (10 ⁶ /ml)	Incubation at 5°C	
	24 h ^a	48 h ^b
1.25	18 ^c	0 ^c
2.5	38 ^{c,d}	6 ^{c,d}
5.0	56 ^d	31 ^{d,e}
10.0	61 ^d	42 ^e
15.0	55 ^d	44 ^e
20.0	58 ^d	41 ^e
S.E. ^f	5.6	6.4

^a (log) linear (P<0.01) and cubic effects (P<0.05).

^b (log) linear (P<0.01) and cubic effects (P<0.1).

^{c,d,e} Means within columns without common superscripts differ (P<0.05).

^f $\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$
(SAS Institute, Cary, NC, USA)

B. Effects of Dilution on Washed Sperm

1. Collection of Source Sample. The second set of the duplicate tubes containing samples prepared in Example 1A were used in this experiment.

2. Methods. The sperm were diluted, incubated and concentrated by centrifugation as in Example 1A. Following centrifugation, 7.1 ml of the supernatant was aspirated from each tube, removing most of the seminal plasma and leaving the sperm in a 900-μl pellet. The sperm were diluted with EYC (see Example 1A) to make 10 × 10⁶/ml or 20 × 10⁶/ml sperm suspensions. The samples were then cooled to 5°C over 90 minutes as in Example 1A.

3. Evaluation of Motility. The samples were warmed and evaluated for progressive motility as in Example 1A.

4. Statistical Analysis. Data were analyzed as in Example 1A. In addition, data in Example 1B were analyzed for incubation concentration at 5°C.

5. Results. Data for washed sperm (Table 2) revealed no significant treatment effects when sperm were evaluated after 24 h. However, after storage for 48 h at 5°C, there were bull, initial dilution, incubation concentration and bull by incubation effects ($P < 0.05$). More sperm remained motile when held at 20×10^6 /ml than at 10×10^6 /ml (31% vs. 20%; $P < 0.05$). Initial dilutions of 1.25, 2.5, and 5×10^6 sperm/ml resulted in lower progressive motility than 10×10^6 sperm/ml ($P < 0.05$), with respective main effect means of 19, 20, 27, and 37% motile sperm.

Table 2. Cumulative effects of washing, dilution, concentration and cooling on progressive sperm motility (%)

Sperm conc (10^6 /ml) during 1 h preincubation at 37°C	Storage at 5°C – Sperm Concentration and Duration			
	24 h		48 h ^a	
	20×10^6 /ml	10×10^6 /ml	20×10^6 /ml	10×10^6 /ml ^b
1.25	45	49	24	15
2.5	51	40	29	11
5.0	54	54	32	21
10.0	51	50	40	34
15.0	60		41	
20.0	55		40	

^a Concentration to 20×10^6 sperm/ml was superior ($P < 0.05$) to 10×10^6 sperm/ml after 48 h storage.

Also, initial dilution to 10×10^6 was superior to lower dilutions ($P < 0.05$).

Pooled standard errors ($\sqrt{\text{error mean square of ANOVA}} \div \sqrt{N}$) were 4.0 for 24 h, and 2.8 for 48 h incubations.

^b Significant (log) linear trend ($P < 0.06$).

C. Conclusion

High sperm dilution and cooling resulted in a substantial reduction in the percentage of motile sperm, regardless of the presence or removal of seminal plasma. However, this dilution

effect was greatly attenuated by concentrating the diluted sperm to $10 \times 10^6/\text{ml}$ and even more, to $20 \times 10^6/\text{ml}$ before storage at 5°C . Sperm from some bulls tolerated dilution better than sperm from other bulls; however, the bull differences found are typical. Extremely dilute sperm might be compromised during sorting, in part, by removal of protective compounds in seminal plasma.

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Example 2

Effects of Equilibration Time Before Freezing Sorted Sperm

Objective: to evaluate the effect of equilibration times (3, 6 and 18 h, 5°C) before freezing on flow-sorted sperm.

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The following experiment was replicated in its entirety using the same bulls:

1. Collection of Source Sample. Sperm of 4 bulls were collected and prepared as described in Example 1A.

15

2. Methods.

a) Staining and Preparation for Sort.

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i) Preparation of Stain Stock Solution: a stock solution of 8.89 mM Hoechst 33342 (bis-Benzimide H-33342; #190305, ICN Biomedicals Inc., Aurora, OH) was prepared in deionized water.

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ii) Sperm Stain Procedure: sperm were diluted in a modified TALP buffer (Table 3) to 400×10^6 sperm/ml. Following dilution, Hoechst 33342 dye was added to the sperm suspensions at a concentration of $224 \mu\text{M}$. After the stain was added to the sperm suspensions, the samples were incubated for 60 minutes at 34°C . Following incubation, sperm were diluted to $100 \times 10^6/\text{ml}$ with TALP containing 2.67% clarified egg yolk and 0.002% food coloring dye (FD&C #40) which quenches the fluorescence of Hoechst 33342 in sperm with compromised cell membranes, allowing them to be gated out during the sorting process. Just prior to flow sorting, samples were filtered at unit gravity through a $40\text{-}\mu\text{m}$ nylon mesh filter to remove any debris and/or clumped sperm.

30

b) Sorting. A two-line argon laser operating at 351 and 364 nm and 150 mW was used to excite the Hoechst 33342 dye. The flow cytometer/cell sorter used was an SX MoFlo® (Cytomation, Inc., Fort Collins, CO, USA) operating at 50 psi. A Tris-based sheath fluid was used, consisting of Tris (hydroxymethyl) aminomethane (Tris; 197.0 mM; #T-1503, Sigma Chemical Co., St. Louis, MO, USA), citric acid monohydrate (55.4 mM; #C-7129, Sigma Chemical Co., St. Louis, MO, USA) and fructose (47.5 mM; #F-0127, Sigma Chemical Co., St. Louis, MO, USA). Baseline antibiotics were also added to the Tris-based sheath fluid consisting of 0.58 g/L of penicillin and 0.05 g/L of streptomycin sulfate.

The sperm were sorted by a process referred to as "bulk sorting" which permits rapid accumulation of large numbers of sperm so that large-scale examples can be done within a reasonable time. The sperm pass through the flow cytometer under the standard operating conditions with the exception that all droplets containing viable sperm were collected into a single tube rather than being sorted into 2 tubes based upon specific characteristics (e.g., sorting by sex-type). Sperm were sorted on the basis of viability; hence, sperm that have compromised plasma membranes were excluded during bulk sorting.

Stained sperm were maintained at $22 \pm 1^\circ\text{C}$ during sorting. Bulk sorted sperm were collected in 50-ml plastic tubes containing 2 ml of 20% egg yolk-Tris extender prepared with 20% egg yolk (vol/vol) in 200 mM Tris, 65 mM citric acid, 56 mM fructose, 50 $\mu\text{g/ml}$ tylosin, 250 $\mu\text{g/ml}$ gentamicin, and 150/300 $\mu\text{g/ml}$ linco-spectin in deionized water. The egg yolk-Tris extender was termed "Tris-A fraction" to denote the lack of glycerol at this point in the procedure. Sperm were collected in tubes to contain 12 ml and approximately 6×10^6 sperm. The sperm were subsequently incubated at 22°C for 1 to 3 h to simulate conditions of a sort based on sex-type.

c) Preparation for Freezing. Following incubation, the sorted sperm were cooled to 5°C over the period of 70 minutes. After cooling, the contents of the two tubes were pooled and transferred to a refrigerated, swinging bucket centrifuge set at 5°C and

centrifuged at $850 \times g$ for 20 minutes. After removing the supernatant, processing continued at 5°C by adding about 150 μl of Tris-A fraction extender to about 150- μl of sperm pellet to bring the sperm concentration to approximately $40 \times 10^6/\text{ml}$. The sperm of individual bulls were pooled and diluted immediately with an equal volume of egg yolk-Tris extender containing 12% (v/v) glycerol ("Tris-B fraction"). The Tris-B fraction was added to the sperm suspension as 2 equal volumes at 15-minute intervals to adjust the final sperm concentration to $20 \times 10^6/\text{ml}$. The final glycerol concentration of the complete egg yolk-Tris extender containing the sperm was 6% (v/v).

d) Equilibration and Freezing. Extended sperm were then packaged into 0.25-ml polyvinylchloride straws to be frozen by routine procedures on racks in static liquid nitrogen vapor. Two straws from each of 4 bulls were frozen after 3, 6 and 18 h of total equilibration time at 5°C .

3. Evaluation of Post-Thaw Motility. Straws were thawed in a 37°C water bath for 30 sec. Blind estimates of progressive motility were made after incubating samples at 37°C for 0, 1 and 2 h post-thawing. Each of two observers estimated progressive sperm motility from each of two straws of semen. These four blind estimates for each experimental unit represent subsampling.

4. Statistical Analysis. Statistically, the subsamples were analyzed as a subplot to the main plot least-squares ANOVAs to analyze effects of any observer and observer $\times$ treatment interaction. N refers to the number of experimental units, not subsamples; standard errors were calculated on the basis of means of the 4 subsamples from error mean squares of ANOVAs and the numbers of experimental units; least-squares means are presented.

Treatment effects were evaluated via separate ANOVAs for each incubation time. The model included bulls as a random effect and equilibration time and observer as fixed effects; the subplot consisted of the observer term and related interactions.

5. Results. The 3- or 6-h equilibration times were superior to 18-h (Table 4), based on the percentage of progressively motile sperm, for 0 and 1 h ($P<0.01$) but not 2 h of post-thaw incubation. Effects of bull were evident at 1 and 2 h incubation times ($P<0.05$), but not at 0 h. There was no significant ($P>0.1$) bull by equilibration time interaction nor was there a significant observer effect for any response.

Table 3. Modified TALP Buffer

Ingredient	Concentration
NaCl	95.0 mM
KCl	3.0 mM
NaHPO ₄	0.3 mM
NaHCO ₃	10.0 mM
MgCl ₂ • 6H ₂ O	0.4 mM
Na Pyruvate	2.0 mM
Glucose	5.0 mM
Na lactate	25.0 mM
HEPES ^a	40.0 mM
Bovine serum albumin ^b	3.0 mg/ml
Gentamycin Sulfate	30.0 µg/ml

^a #H3375, Sigma Chemical Co., St. Louis, MO, USA

^b #US70195, fraction V; Amersham/Life Science, Cleveland, OH, USA

Table 4. Effect of pre-freeze equilibration time on post-thaw progressive motility (%)

Equilibration at 5°C	Post-thaw incubation at 37°C		
	0 h	1 h	2 h
3 h	41 ^a	36 ^{a,b}	16
6 h	41 ^a	37 ^a	18
18 h	35 ^b	31 ^b	12
S.E. ^c	1.5	0.8	2.0

^{a,b} Within columns, means without common superscripts differ ($P < 0.05$), Tukey's HSD.

^c Pooled standard errors,

$$\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$$

6. Conclusion. The results indicated no differences in post-thaw sperm motility between 3 and 6 h of total equilibration time at 5°C, but there was a significant decline in sperm motility following 18 h of equilibration at 5°C before freezing. The 3- to 6-h range permits pooling 2 consecutive 3-h sorting batches for freezing sperm without decreasing post-thaw motility.

As the bull by equilibration-time interaction was not significant, 3 to 6 h equilibration was adequate, with the caveat that only 4 bulls were used. The optimum equilibration time for a minority of bulls is expected to be >6 h.

Example 3

Effects Of Stain Concentration And Laser Power on Sorted Sperm

Objective: to evaluate the effects of Hoechst 33342 dye concentration in combination with laser intensity on flow-sorted sperm.

5

1. Collection of Source Sample. Sperm of 6 bulls were collected and prepared as described in Example 1A.

2. Methods.

10

a) Experimental Design. One ejaculate (2 bulls) and 2 ejaculates on different days (4 bulls) were used in a 2 by 2 design plus control.

15

b) Staining and Sorting. Staining, preparation for sorting and sorting sperm were achieved as described in Example 2 except that the Hoechst 33342 dye was added to sperm suspensions at a final concentration of 149 μM or 224 μM ; and sperm were bulk-sorted with the laser operating at 100 mW or 150 mW of incident power. Bulk-sorted sperm were collected into 50-ml plastic tubes as described in Example 2. Four tubes containing approximately 15×10^6 total sperm/tube were collected over 1 h for each bull. The sorted sperm were incubated for 1 h at 22°C to simulate a longer sorting time.

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c) Preparation for Freezing. Following incubation, the sperm were cooled as in Example 2. The sperm were then concentrated by centrifugation at 5°C at $850 \times g$ for 20 minutes. After removing the supernatant, 150 μl of Tris-A fraction extender was added to each 150- μl sperm pellet at 5°C. All of the sperm pellets were suspended by gentle repeated aspiration and the sperm of individual bulls were pooled. Tris B-fraction extender was added stepwise as described in Example 2. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in Tris extender containing 6% glycerol and cooled to 5°C while the bulk-sorted sperm were being prepared.

30

d) Equilibration and Freezing. The control and sorted sperm were packaged into 0.25-ml polyvinylchloride straws as described in Example 2, equilibrated at 5°C for 3 h and then frozen conventionally.

5 3. Evaluation of Post-Thaw Motility. Straws were thawed and evaluated as described in Example 2.

4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via ANOVA. The model included dye
10 concentration, laser intensity and bulls in the main plot, and observer and related interactions in the subplot. Bulls were considered a random effect and the other factors as fixed.

5. Results. Bull effects were significant for percentages of progressively motile sperm immediately after thawing ($P < 0.1$) and after 1 h and 2 h of incubation at 37°C ($P < 0.05$).
15 There was no effect of dye concentration or bull by dye concentration on sperm motility at any incubation time. With bulls considered as a random effect, 150 mW of laser power resulted in lower post-thaw motility of sperm than 100 mW at 0 h of incubation ($P < 0.1$), but not at other incubation times (Table 5). If bulls are considered as fixed effects, 150 mW of power resulted in lower sperm motility than 100 mW ($P < 0.05$) at all 3 incubation times. There was an effect of
20 bull by laser power ($P < 0.05$) on sperm motility at 1 h, but not at 0-h or 2-h incubation times. Also, the higher laser power resulted in lower sperm motility than the control ($P < 0.05$) at 0- and 1-h incubation times (Table 5). There was a significant observer effect at 1-h, but not at 0-h or 2-h, incubation times. There was no observer by treatment interaction ($P > 0.1$).

25

30

Table 5. Effects of laser intensity and dye concentration on post-thaw motility (%).

Main effect means	Incubation at 37°C		
	0 h	1 h	2 h
Control	49	44	33
<u>Dye Concentration</u>			
149 µM	41	39	30
224 µM	42	39	30
<u>Laser Intensity</u>			
100 mW	46	42	33
150 mW	38 ^a	35 ^b	27
S.E. ^c	2.2	1.2	1.3

^a Significant main effect (P<0.1) and differs from control (P<0.05).

^b Differs from control (P<0.05).

^c Pooled standard errors, $\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$

6. Conclusion. Percentages of progressively motile sperm post-thaw were diminished by the staining and sorting process. Higher laser intensity was more damaging than the lower laser intensity. There was no effect of dye concentration on post-thaw sperm motility. Thus, excitation of the sperm-bound Hoechst 33342 dye at lower laser intensities is less damaging and that staining sperm at the higher dye concentration had no detrimental effect on post-thaw motility. The damage observed was presumably to the sperm-motility apparatus.

Example 4

Evaluation of Pre-Sort Staining Procedures and Selection of Extenders for the Cryopreservation Of Sperm

Objective: (1) to evaluate three pre-sort treatments for sperm; and, (2) and to evaluate sheath
fluid and extender combinations for the cryopreservation of flow-sorted sperm.

The following experiment was replicated in its entirety:

1. Collection of Source Sample. Sperm from 4 bulls were collected and prepared as described in Example 1A.

2. Methods.

a) Experimental Design. A 3 (pre-sort treatments) by 3 (extenders) by 2 (sheath fluids) by 4 (bulls) by 2 (observers) factorial experiment was designed to determine the best procedure to hold sperm prior to sorting, and to evaluate three extenders for cryopreserving the sorted sperm. .

b) Sample Preparation and Staining. Freshly collected sperm from each of 4 bulls were treated as follows:

(1) diluted to 400×10^6 /ml in modified TALP (see Example 2, Table 3) and stained for 1 h at 34°C before bulk-sorting ("Dilute - 0 h");

(2) incubated neat at 22°C for 3 h before dilution, staining and sorting ("Neat - 3 h"); or,

(3) diluted and stained as "Dilute-0 h" and then incubated at 22°C for 3 h before bulk-sorting ("Diluted - 3 h").

c) Extenders. The following freezing extenders were compared: EYC (see Example 1) containing 7% glycerol, egg yolk-Tris (see Example 2) containing 6% glycerol, and egg yolk-TES-Tris (TEST) containing 5% glycerol. EYC "A Fraction" refers to the EYC extender containing no glycerol, and EYC "B Fraction" refers to EYC extender containing twice the final, desired glycerol

concentration (i.e., 14%). Thus, when EYC A and B fractions are combined in equal volume, the final EYC extender contains 7% glycerol. Tris A and B fractions are similarly named, and described in Example 2. TEST extender is prepared as a complete extender containing 5% glycerol; hence, there were no "A" and "B" fractions for TEST.

d) Sheath Fluid. Sheath fluid was either 98.6 mM sodium citrate dihydrate (#S279-3, Fisher Scientific, Fair Lawn, NJ) or Tris as described in Example 2. Both types of sheath fluid were adjusted to pH 6.8; osmolality was about 270 to 280 mOsm/kg. Tris sheath fluid was used to collect sperm that were later extended in egg yolk-Tris and TEST freezing extenders. Sheath fluid containing 98.6 mM sodium citrate dihydrate was used to collect sperm to later be extended in EYC freezing extender.

e) Sorting. Approximately 58×10^6 sperm for each combination of pre-sort treatment, sheath fluid and extender were bulk-sorted as described in Example 2 using 150 mW of incident laser power. For each sort, sperm were collected over approximately 1 h. After sorting, the samples were incubated at 22°C for 2 h to simulate a 3 h sort.

f) Preparation for Freezing. Following incubation, the sperm were cooled as described in Example 2. After cooling, the samples were centrifuged at 5°C at $850 \times g$ for 20 min. Each sample comprised about 28 ml total volume and was contained in a 50-ml plastic tube

After the supernatant was removed, the sperm were returned to a 5°C cold room for extension. Samples were extended to 40×10^6 /ml by depositing 131 μ l of the sperm suspension into 69 μ l of A-fraction EYC, A-fraction egg yolk-Tris, or TEST extender. Immediately, suspensions were adjusted to 20×10^6 sperm/ml with the addition of the matched glycerol containing extender (i.e., B-fraction EYC, B-fraction Tris) or TEST. B-fraction extenders were added to their respective samples stepwise (2X) at 15-min intervals as described in Example 2.

The TEST was added to sperm stepwise in the same manner as B-fraction EYC and Tris extenders.

g) Equilibration and Freezing. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for 3 h at 5°C and then frozen in static liquid nitrogen vapor.

3. Evaluation of Post-Thaw Motility. Thawing and post-thaw evaluations of sperm were done as described for Example 2.

4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each post-thaw incubation time. The main plot included pre-sort treatment, extenders, and bulls; the subplot consisted of observers and associated interactions. Bulls were considered a random effect, and the other factors, fixed. The entire experiment was replicated twice. Tukey's HSD test was used to separate means.

5. Results. Post-thaw progressive motility of bulk-sorted sperm was affected ($P < 0.05$) by extender and bulls at each post-thaw incubation time and by pre-sort procedure at 0 h of incubation (Table 6). There were no differences due to sheath fluids ($P > 0.05$). At 0-h post-thaw incubation, use of the neat-3h treatment resulted in more motile sperm after freezing and thawing than the other 2 pre-sort staining treatments ($P < 0.05$; Table 6). However, pre-sort procedures were not statistically significant after post-thaw incubation of sperm for 1 or 2 h with bulls considered as a random effect. Importantly, at these 2 incubation times, there were significant pre-sort treatment by bull interactions ($P < 0.05$). Furthermore, pre-sort treatment would have been a significant effect at all post-thaw incubation times had bulls been considered as fixed effects.

Immediately after thawing (0 h), TEST was the best extender, but after 1 or 2 h of incubation of 37°C, Tris was the best extender. Importantly, there was no pre-sort treatment by extender interaction for any response. There were observer effects ($P < 0.01$) at all incubation

times, but no observer by treatment interactions. There was a bull by extender interaction (P<0.05) at all 3 incubation times.

Table 6. Main effects of pre-sort treatment and freezing extenders on post-thaw progressive motility (%)

Pre-sort procedure	Extender	Incubation at 37°C		
		0 h	1 h	2 h
Dilute – 0 h	Mean	39 ^a	32	22
Neat – 3 h	Mean	43 ^b	36	25
Dilute – 3 h	Mean	38 ^a	31	19
Mean	EYC	36 ^a	29 ^a	17 ^a
Mean	Tris	40 ^b	39 ^b	29 ^b
Mean	TEST	44 ^c	33 ^c	20 ^a
S.E. ^d		0.8	0.8	0.7

^{a,b,c} Means within columns, within main effects, without common superscripts differ (P<0.05).

^d Pooled standard errors = $\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$

6. Conclusion. This study showed that holding sperm neat for 3 h before dilution, staining and sorting was better than immediate dilution and staining 0 h or 3 h later. Thus, by 3 h into the sort, it is best to continue with a new aliquot of the original ejaculate that was held neat 3 h and then stained, rather than continuing with the original sample of sperm stained and held at 400 x 10⁶ sperm/ml.

Even though TEST extender provided higher post-thaw motility at 0 h, Tris was the superior extender when sperm were stressed by incubation at 37°C. Either sheath fluid worked equally well for each extender. Based on these results, we have incorporated the use of Tris sheath fluid in combination with Tris freezing extender into our standard operating procedure.

Example 5

Effects Of Extender Additives on Sorted Sperm

Objective: to evaluate the effect of adding sodium dodecyl sulfate ("SDS") to the freezing extender on flow-sorted sperm.

5

A. *Evaluation of Effect of Concentration of SDS in Freezing Extender*

1. Collection of Source Sample. Sperm of 6 bulls were collected and prepared as described in Example 1A.

10

2. Methods. Sperm from each of 6 bulls were extended to 20×10^6 /ml in 20% whole egg Tris ("WET") extender containing 0, 0.03, 0.06, 0.09, or 0.12 percent SDS, packaged into straws and frozen. WET extender was prepared using 3.028 g of Tris[hydroxymethyl] aminomethane, 1.78 g of citric acid monohydrate, and 1.25 g of fructose per 100 ml of double distilled water, to which 20% whole egg (vol/vol) was added. The WET extender was prepared at a pH of about 7.0 and contained a final glycerol concentration of about 6% (vol/vol). The WET extender also contained 1000 IU of penicillin "G" sodium and 100 µg of streptomycin sulfate/ml.

15

3. Results. The respective means (n = 1 sample from each of 6 bulls) were 51, 51, 50, 51, and 48% progressive motile sperm approximately 10 minutes post-thaw. Based on these results, 0.06 percent SDS was used in Example 5B.

20

B. *Evaluation of the Effects of 0.06 Percent SDS in Various Freezing Extenders on Post-Thaw Motility of Flow-Sorted Sperm*

25

1. Collection of Source Sample. Sperm of 8 bulls were collected and prepared as described in Example 1A.

30

2. Methods. Post-thaw motility was studied for sperm frozen in egg yolk-Tris (see Example 2) and WET extenders (see Example 5A) with and without 0.06% SDS. Final glycerol content for both extenders was 6%.

a) Staining, Preparation for Sort, Sorting. Stained sperm samples were prepared from an ejaculate from each of 8 bulls as described in Example 2. Stained sperm were bulked-sorted using Tris sheath fluid as described in Example 2 except that the sort was achieved using 135 mW of incident laser power. Sorted sperm were collected in a 50-ml plastic tube containing 2 ml of A-fraction freezing buffer for each extender; 15×10^6 total sorted sperm (25 ml) for each treatment were collected and incubated for 1 h at 22°C to simulate longer sorting.

b) Preparation for Freezing. Diluted sperm were then cooled to 5°C over 90 minutes. An equal volume of appropriate B-fraction extender was added stepwise (2x) at 15-minute intervals to each 50-ml plastic tube containing sorted sperm. Aliquots of 25 ml/extender treatment were concentrated by centrifugation for 20 minutes at 850 x g in a refrigerated centrifuge. The supernatant was removed leaving a 600 µl sperm pellet, which was suspended by gentle vortexing for 15 seconds. No additional extender was added to the sperm pellet since the suspension containing the pellet already contained glycerol. The concentration of the sperm suspension was approximately 20×10^6 /ml. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in egg-yolk-Tris extender containing 6% glycerol. The control was placed in a 5°C cold room while bulk-sorting occurred.

c) Equilibration and Freezing. All control and bulk-sorted sperm were packaged and frozen at the same time. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for about 3 h to about 6 h at 5°C and then frozen in static liquid nitrogen vapor.

3. Evaluation of Post-Thaw Motility. Thawing and post-thaw evaluations of sperm were done as described for Example 2 with the exception that progressive motility was evaluated 0.5 and 2.0 h after incubation.

4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each incubation time; the model included bull and extender in the main plot and observer and

related interactions in the subplot. Differences in means were determined by the least significant difference test.

5. Results. Extender affected ($P<0.05$) progressive motility of sperm after 0.5 or 2 h post-thaw incubation (Table 7). At 0.5 h, WET plus SDS resulted in lower motility than Tris with SDS. At 2 h, all treatments with bulk-sorted sperm were worse than the non-sorted control sperm. There were significant bull and observer effects ($P<0.01$) at both incubation times, but no observer by treatment interactions.

Table 7. Effect of extender on post-thaw progressive motility (%)

Extender	Incubation at 37°C	
	0.5 h	2 h
Tris (non-sort)	42 ^a	41 ^a
Tris w/o SDS	40 ^{a,b}	35 ^b
Tris w/SDS	42 ^a	37 ^b
WET w/o SDS	40 ^{a,b}	35 ^b
WET w/SDS	38 ^b	35 ^b
S.E. ^c	1.0	1.2

^{a,b} Means within columns without common superscripts differ ($P<0.05$).

^c $\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$

6. Conclusion. The inclusion of SDS in Tris or WET extenders did not benefit sperm quality as determined by visual estimates of motility after thawing. Also, results using WET and Tris extenders were similar; hence, WET appeared as efficacious as Tris for cryopreserving sorted bovine sperm.

Example 6

Quality of Sperm Sexed by Flow Sorting for Field Trials

Objective: to evaluate post-thaw quality of sorted sperm based on acrosomal integrity.

- 5 1. Collection of Source Sample. Sperm of 3 bulls were collected and prepared as described in Example 1A.

2. Methods. Sorted and non-sorted control sperm from the same ejaculate were stained, processed, and sorted as described in Example 2 except the sperm were sorted for sex-
10 type at a 90% purity level. Sorted sperm were collected to a volume of approximately 20 ml and were cooled to 5°C for 90 minutes (0.2°C/min). After cooling, an equal volume of egg yolk-Tris B extender (see Example 2) was added to the sorted sperm in 2 equal volumes at 15-minute intervals. Centrifugation and aspiration of the supernatant were achieved as described in Example 5. After centrifugation and aspiration, egg yolk-Tris extender containing 6% glycerol
15 (v/v) was added to the sperm pellet to bring the concentration of sperm to about 20×10^6 /ml. Freezing and thawing were done as described in Example 2 except that equilibration time was about 3 h.

3. Evaluation of Post-Thaw Motility. Visual estimates of the percentage of
20 progressively motile sperm at 37°C were made approximately 10 minutes after thawing. The acrosomal integrity of sperm was assessed using differential interference-contrast microscopy (x1000) after 2 h of incubation at 37°C. Sperm were treated with 40 mM sodium fluoride, a wet was smear made, and 100 sperm per treatment were examined. Acrosomes were classified as: (a) intact acrosome, (b) swollen or damaged acrosome, or (c) missing acrosome (non-intact).

- 25 4. Statistical Analysis. The data analyzed were from 19 different freeze dates balanced across 3 bulls used in field trials. Treatment effects (sort vs. control) were evaluated via analysis of variance with bulls as a fixed effect.

5. Results. The percentage of progressively motile sperm post-thaw was
30 significantly higher ($P < 0.05$) for non-sorted sperm (50%) than for sorted sperm (46%; Table 8), despite removal of dead sperm during sorting. However, the percentage of sperm with an intact

acrosome was not different. Sorting increased the percentage of sperm missing an acrosome, but also reduced the percentage of sperm with a damaged acrosome, relative to control sperm ($P<0.05$). There were significant differences among bulls for percent of intact acrosomes ($P<0.05$), percent of non-intact acrosomes ($P<0.01$), and post-thaw progressive motility ($P<0.01$). There was a bull by sorting effect for post-thaw motility ($p<0.01$) but not for the other responses. From bulls A and B, differences in post-thaw motility between sorted and unsorted sperm were near zero; for bull C, sorted sperm were 10 percentage points (19%) lower in motility than control sperm.

Table 8. Effect of sorting on post-thaw motility (%) and acrosomal status (%).

	Acrosomal status			Post-thaw motility
	Intact	Damaged	Non-intact	
Control	64 ^a	20 ^a	15 ^a	50 ^a
Sorted	65 ^a	14 ^b	21 ^b	46 ^b

^{a,b} Column means with different superscripts differ ($P<0.05$).

6. Conclusion. Visual estimates of progressive motility for sorted, frozen sperm on average were slightly lower (4 percentage points; 8%) than for control sperm, although this difference was larger for one bull. These evaluations were made approximately 10 minutes after thawing. The small average difference is consistent with that for non-intact acrosomes after 2 h of incubation. Sperm with a damaged or missing acrosome are likely to be immotile. The increased percentage of sperm with a non-intact acrosome, for sorted samples, indicates damage associated with sorting or with cryopreservation before or after actual sorting. Presumably, sorting converted damaged acrosomes to missing acrosomes. Based on standard procedures for evaluation of sperm quality, there is no basis for assuming that fertilizing potential of these flow-sorted sperm should be severely compromised for most bulls.

Example 7

Sex-Selection and Cryopreservation of Bull Sperm Using 20% Egg Yolk-Tris Extender

Objective: to provide a protocol for the cryopreservation of flow-sorted bull sperm.

5 1. Collection and Ejaculate Assessment. Collect and prepare ejaculates as described in Example 1A. Select ejaculates from those bulls with >75% morphologically normal sperm. Visually estimate the percentage of progressively motile sperm (ejaculates that have progressive motility >60% are best for sorting). Add antibiotics to raw semen as follows: tylosin at a final concentration 100 µg/ml, gentamicin at a final concentration of 500 µg/ml, and linco-spectin at a
10 final concentration of 300/600 µg/ml.

 2. Staining and Preparation for Sort. Following the addition of the antibiotics to the raw semen sample, allow 15-20 minutes before staining. Stain samples as described in
15 Example 2.

 3. Sorting. Sort for both X- and Y- type sperm, setting the sorting gates for 90% purity. Sort sperm into 50-ml Falcon tubes containing 2 ml 20% egg yolk-Tris A-fraction extender (see Example 2) until each tube contains a maximum of 20 ml total volume (or a maximum of 2 h per sort) and final sorted sperm concentration is 6×10^5 /ml. Note that
20 additional 20% egg yolk-Tris-A fraction catch buffer must be added after the sort and prior to cooling so that the final percentage of egg yolk is at least 3%.

 4. Preparation for Freezing. Following the sort, cool the sorted samples to 5°C over a period of 90 minutes. After cooling, add 20% egg yolk-Tris B-fraction extender (see Example
25 2) stepwise (2X) at 15 minutes intervals. The final volume of Tris B-fraction extender added to the sperm sample should be equal to the volume of Tris A-fraction extender. The total volume of sperm sample after the Tris B-fraction extender is added should not exceed 27 ml total volume.

 After the Tris B-fraction extender is added to the sperm sample, concentrate the
30 sample by centrifugation for 20 minutes at $850 \times g$. Aspirate the supernatant leaving approximately 150 µl sperm pellet. Resuspend the sperm and pool the sperm for each individual bull.

5. Freezing. Add complete egg yolk-Tris extender (6% glycerol) to achieve a final sperm concentration of 20×10^6 /ml. Package the extended sperm into 0.25-ml polyvinylchloride straws for freezing as described in Example 2.

5

Example 8

Evaluation of the Fertility of Flow-sorted, Frozen Bull Sperm in Field Studies

MATERIALS AND METHODS

10 Semen Collection and Processing

Semen from young bulls of unknown fertility was collected via artificial vagina (see Example 1A). After determining sperm concentration with a spectrophotometer and subjective evaluation of progressive sperm motility, semen was processed and sorted as described in Example 2 except that the sperm were sorted by sex-type at 90% purity using a laser incident power of about 135 to about 150 mW. Processing and freezing was achieved as in Example 2 except that the equilibration time was about 3 h. Cornell Universal Extender (Seidel GE Jr., Theriogenology 1997; 48:1255-1264) was used for liquid semen in field trials 1, 2, and 3. For frozen semen in field trials 2 and 3, the extender used was 2.9% Na citrate + 20% egg yolk with a final glycerol concentration of 7% (see Example 1). For field trials 4 through 11, sperm were frozen in a Tris-based extender composed of 200 mM Tris, 65 mM citric acid, 56 mM fructose, 20% egg yolk, and a final glycerol concentration of 6% (see Example 2). The sheath fluid used in the flow cytometer was 2.9% Na citrate (see Example 4) for trials 1, 2, and 3, and a Tris buffer for the remaining trials (see Example 2).

Sperm were packaged in 0.25-ml French straws in columns as small as 50 μ l in the center of the straw. To minimize dilution effects, low volumes were used so there were at least 10^7 sperm/ml. In most trials, a column of extender without sperm was aspirated into the straw first to wet the cotton plug, followed by a small column of air, and then the sexed sperm. When sperm were frozen, one straw from each batch was thawed in 35°C water for 30 sec for quality control, and batches with less than 25% progressive motility post thaw were discarded. A sample of sexed sperm from each batch was sonicated and analyzed by flow cytometry to determine the accuracy of sexing.

Heifer Management and Artificial Insemination

The heifers used were in 6 widely scattered production units with different management practices. Seasonal and breed differences contributed further to the heterogeneity of the experiments (Table 9). Insofar as possible, treatments and controls were alternated systematically within bulls within inseminators as heifers entered the insemination facilities.

Estrus was synchronized in one of 4 ways (Table 9): (1) 500 mg of melengesterol acetate (MGA) fed daily in 2.3 kg of grain for 14 days followed by an i.m. injection of 25 mg prostaglandin F₂α (Lutalyse, Upjohn, Kalamazoo, MI, USA) 17, 18 or 19 days after the last day of feeding MGA (MGA/PG); (2) a single injection of 25 mg of prostaglandin F₂α (PG); (3) 20 or 25 mg of prostaglandin F₂α injected i.m. at 12-day intervals (PG/PG) or (4) 50 or 100 µg of GnRH injected i.m., followed by 25 mg of prostaglandin F₂α 7 days later (GnRH/PG).

Heifers were inspected visually for standing estrus mornings and evenings, but inseminated only in the evenings after 16:00, approximately ½ or 1 day after onset of estrus.

Insemination was either into the uterine body conventionally, or half into each uterine horn using atraumatic embryo transfer sheaths (IMV, Minneapolis, MN, USA). In the latter case, semen was deposited past the greater curvature of the uterine horn as far anterior as could be accomplished without trauma, identically to nonsurgical embryo transfer. In most cases, semen was deposited between the anterior third and mid-cornua.

Most experiments included a frozen sperm control inseminated into the uterine body with 20 or 40 x 10⁶ sperm/dose from the same bulls used for sperm sorted for sex-type ("sexed"). This control served as a composite estimate of the intrinsic, normal fertility of the heifers under the specific field-trial conditions as well as the fertility of the bulls used and the skills of the inseminators. Some trials also included a low-dose, unsexed control group. Sometimes numbers of control inseminations were planned to be ½ or ⅔ the number used for each treatment to obtain more information on sexed sperm. Frozen sexed and control sperm were thawed for 20 to 30 sec in a 35 to 37°C water bath. Various other details are summarized in Table 9.

Pregnancy was diagnosed by ultrasound 28 to 37 d post insemination and/or 56 to 92 d post-insemination, at which time fetal sex was determined in most trials, as described in Curran, S., Theriogenology 1991; 36:809-814, without the operator's knowing insemination treatments or controls. Sexes of calves born were nearly identical to the fetal-sex diagnosis. Data were

analyzed by single-degree-of-freedom Chi square corrected for continuity; 2-tail tests were used unless 1-tail is specified. Fewer than 5% of the inseminations were culled due to errors of insemination treatment, frank infection of the reproductive tract, failure to traverse the cervix, etc. Decisions to cull animals from experiments were made shortly after insemination and were
5 never based on the pregnancy diagnosis.

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Table 9. Procedural details of field trials

	Trial	Insemination dates	Breeds of heifers	Bulls used	Inseminator s	Estrus synchroni- zation	Comments
5	1	5/20-23, 1997	Angus	N1, N2, AN4	A, B	MGA/PG	Included low-dose controls
	2	2/18-5/22, 1998	Angus crossbred	N3, N4, N5, N6	C, D	PG/PG	Low dose but no normal- dose controls; some heifers pregnant and aborted when synchronized
10	3	6/2-6/5, 1998	Angus	AN4, AN5, N7, N8	B, D	MGA/PG	
	4	2/10-13, 1999	Holstein	J2, J4	C, D	PG	Very severe mud, snow, wind, and cold, driving rain
15	5	2/24-26, 1999	Holstein	J2, J4, J5	C, B, D	PG/PG	
	6	4/14-16, 1999	Holstein	J2, J3, J4, J5	C, D	PG	Some heifers were reproductive culls
20	7	4/27-5/1, 1999	Hereford& Angus crossbred	AN1, AN4	C	MGA/PG	Semen for 1 bull shipped 6 h before sorting; severe weather
	8	4/21-23, 1999	Angus crossbred	H1, H2	E	MGA/PG	Feedlot heifers
25	9	5/5-8, 1999	Red Angus	AR1, AR2	C, F	MGA/PG	
	10	5/31-6/2, 1999	Angus	AN4, AN7, AN8	B, D	GnRH/ PG	
30	11	7/28-30, 1999	Holstein	H2, H3	C, D	PG/PG	First replicate available in a much larger trial

RESULTS AND DISCUSSION

The data presented are from 11 consecutive, heterogeneous field trials, constrained by logistical aspects of the studies, such as having to match bulls to genetic needs of the herds, unavailability of fertility information on bulls, limited numbers of heifers, unavailability of the same inseminators across trials, severe weather in some trials, limited amounts of sexed semen in early trials, 2 sets of heifers in which some turned out to be pregnant up to about 55 days at the time of estrus synchronization, etc. Up to 4 bulls and 3 inseminators were involved with each trial; this enabled us to sample populations to ensure that results applied to more than one bull or technician; however, insufficient data were produced to evaluate bull-to-bull differences in fertility rigorously.

Most sets of heifers were from breeding herds located 140 to 250 km from our laboratory. There were no significant differences in pregnancy rates between inseminators in any trial, but numbers of breedings per inseminator were low, and differences likely would be detected with larger numbers of inseminations.

Estrus synchronization methods were not compared within trials, so it was not possible to compare pregnancy rates among these methods. Pregnancy rates appeared to be satisfactory for all four synchronization procedures used.

Since inseminations were done once a day, heifers in estrus evenings were inseminated approximately 24 h after estrus was detected. The pregnancy rate for these heifers with sexed sperm pooled over all trials was 203/414 (49.0%), which was not significantly different ($P>0.1$) from that of heifers in estrus mornings and thus inseminated half a day after estrus detection 266/586 (45.4%). This tendency for higher fertility with later insemination is in agreement with findings from other research that it is preferable to inseminate later than normally recommended with lower fertility bulls, when low sperm numbers are used, or when conditions are otherwise suboptimal.

Pregnancy rates by treatments and, when available, fetal or calf sex are presented in Tables 10 to 20. The objective was to obtain female offspring, except in trial 8; accuracy was 95%, 83%, 90%, 83%, 82%, and 94% in Trials 1, 3, 8, 9, 10, and 11, respectively. In the remainder of the trials, fetal or birth sexes were not available because of timing of pregnancy diagnosis, unavailability of persons skilled in sexing fetuses, and/or because calves have not yet

been born. This was not a major concern because the main objective of this research was to determine fertility of flow-sorted sperm inseminated at low doses.

The accuracy of sexing can be adjusted to virtually any level desired between 50 and 95+% by adjusting the sorting parameters. However, higher accuracy results in lower numbers of sperm sorted per unit time, particularly for Y-chromosome sperm. 90% accuracy is sufficient for routine work.

The main findings from each field trial will be summarized in turn. Note that total sperm numbers are given in table headings; numbers of progressively motile sperm usually were 30 to 50% of these values. Field trial 1 (Table 10) confirmed that pregnancy rates with uterine horn insemination using low numbers of unsexed sperm were similar to controls with normal sperm numbers. The day 64 to 67 pregnancy rate with unfrozen sexed sperm (42%) was 12 percentage points below the unsexed liquid control with sperm diluted, stained, and centrifuged identically to the sorted sperm. Accuracy of sexing was 95%; the sex of calves born from sexed sperm matched the sex diagnosis of fetuses exactly; there was one mistake in sexing fetuses of controls. There were no abortions between 2 months of gestation and term, and all 19 calves from the sexed sperm treatment were normal and survived. For the sexed semen treatment, the 2-month pregnancy rates for bulls N1, N2, and N3 were 41, 44, and 40%, respectively; 39% (13/33) of heifers in estrus in the morning and 50% (6/12) in estrus in the evening became pregnant.

Table 10. Results of field trial 1 -- Angus heifers in Wyoming, 1997

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 33	No. pregnant day 64 to 67	No. ♀ calves
Sexed, 5°C/horns	3 x 10 ⁵	45	20 (44%)	19 (42%)	18 (95%) ^a
Control, 5°C/horns	3 x 10 ⁵	28	15 (54%)	15 (54%)	5 (53%) ^b
Frozen, control/body	40 x 10 ⁶	29	16 (55%)	15 (52%)	11 (73%) ^{a,b}

^{a,b} Sex ratios without common superscripts differ (P<0.02).

Field trial 2 (Table 11) provided the first evidence that results with sexed, frozen sperm are similar to sexed, unfrozen sperm if adjustment is made for numbers of sperm killed during cryopreservation. There also was no difference in pregnancy rates between sexed sperm stored at 5 versus 18°C. Pregnancy rates at 2+ months after insemination for sexed semen from individual bulls ranged from 22 to 42% pregnant ($P>0.05$). Embryonic loss between 1 and 2 months of gestation was very similar for sexed and control pregnancies. Calving data were available from 39 heifers from this trial; each of these heifers (30 sexed pregnancies, 9 controls) pregnant at 2 months calved after a normal-length gestation.

Table 11. Results of field trial 2--Crossbred beef heifers in Colorado, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 35 ^a	No. pregnant day 59 to 92 ^a
Control, 5°C/horns	5×10^5	58	27 (47%)	24 (41%)
Sexed, 5°C/horns	5×10^5	51	17 (33%)	16 (31%)
Sexed, 18°C/horns	5×10^5	46	16 (35%)	12 (26%)
Sexed, frozen/horns	1×10^6	87	29 (33%)	28 (32%)

^a No significant differences, χ^2 .

Field trial 3 (Table 12) confirmed that sexed, frozen sperm results in reasonable pregnancy rates. The accuracy of sexing sperm was confirmed again; however, there were 4 errors in sexing fetuses relative to the calves born; the actual sexes of calves born are presented. Again, there were no abortions between 2 months of gestation and term. Pregnancy rates averaged over sexed, unfrozen and sexed, frozen sperm for bulls N8, N9, AN5, and AN4 were 24, 31, 50, and 60%, respectively ($P<0.1$).

Table 12. Results of field trial 3 -- Angus heifers in Wyoming, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 62 to 65	No. ♀ calves
Sexed, 18°C/horns	5 x 10 ⁵	37	11 (30%) ^a	10 (91%) ^c
Sexed, frozen/horns	1 x 10 ⁶	35	18 (51%) ^{a,b}	14 (78%) ^c
Frozen, control/body	40 x 10 ⁶	37	27 (73%) ^b	16 (59%) ^d

^{a,b} Means without common superscripts differ P<0.05).

^{c,d} The percentage of ♀ calves from the sexed treatments (83%) differed from the control group, P<0.05, 1-tail, χ^2 .

Field trials 4, 5, and 6 (Tables 13, 14, 15) were done at the same location with 3 different groups of heifers. Unfortunately, it was not possible to replicate each trial similarly due to vagaries of field trials, such as scheduling personnel, availability of sexed semen from each bull, etc. The widely different pregnancy rates between trials 5 and 6 illustrate that conditions were different among trials. Some of the heifers in trial 6 were available because they failed to get pregnant after a month of natural mating. Under conditions of these trials, pregnancy rates were very similar between 1.5 and 3.0 x 10⁶ sexed, frozen sperm/dose. Furthermore, there was no advantage to uterine-horn insemination. There were no significant differences (P>0.05) in pregnancy rates among bulls except in Trial 5 in which the pregnancy rate of J2, 20/28 (71%), was higher than that of J4, 15/39 (38%) (P<0.05). This difference was not consistent from trial to trial, as J4 had numerically but not significantly (P>0.1) higher pregnancy rates than J2 in Trials 4 and 6.

Table 13. Results of field trial 4 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 33	No. pregnant day 64 to 67*
Sexed, frozen/body	1.5×10^6	55	36 (65%) ^{a,b}	36 (65%) ^{a,b}
Sexed, frozen/body	3×10^6	52	27 (52%) ^a	26 (50%) ^a
Control, frozen/body	20×10^6	55	45 (82%) ^b	43 (78%) ^b

^{a,b} Means without common superscripts differ ($P < 0.01$).

* Six heifers pregnant at d 30 to 33 were sold before the second pregnancy diagnosis; these were assumed to have remained pregnant.

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Table 14. Results of field trial 5 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 35 ^a	No. pregnant day 60 to 62 ^a
Sexed, frozen/body	1.5×10^6	23	12 (52%)	12 (52%)
Sexed, frozen/body	3.0×10^6	25	15 (60%)	14 (56%)
Sexed, frozen/horns	1.5×10^6	25	15 (60%)	12 (48%)
Sexed, frozen/horns	3.0×10^6	25	17 (68%)	15 (60%)
Control, frozen/body	20×10^6	30	20 (67%)	19 (63%)

^a No significant differences.

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Table 15. Results of field trial 6 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 34	No. pregnant day 60 to 63
5 Sexed, frozen/body	1.5×10^6	27	11 (41%) ^a	9 (33%) ^a
Sexed, frozen/body	3.0×10^6	25	10 (40%) ^a	9 (36%) ^a
Sexed, frozen/horns	1.5×10^6	24	8 (33%) ^a	7 (29%) ^a
Sexed, frozen/horns	3.0×10^6	24	10 (42%) ^a	8 (33%) ^a
Control, frozen/body	20×10^6	24	18 (75%) ^b	17 (71%) ^b

10 ^{a,b} Means without common superscripts differ ($P < 0.05$).

For trial 7 (Table 16), only one inseminator was available due to rescheduling. This is the only trial that showed a convincing advantage of uterine-horn over uterine-body insemination. For this inseminator under the conditions of the trial, 55% more heifers (22 percentage points) became pregnant with sexed, frozen semen inseminated into the uterine horns than into the uterine body. The true difference could be smaller because there are wide confidence intervals on these means. In all the other trials (5, 6, 9, and 11) in which body- and horn-insemination were compared, pregnancy rates were very similar for both methods for this technician as well as for other technicians.

20 Semen from one of the bulls used in Trial 7 was shipped without dilution from Montana by air in an insulated box at $\sim 20^\circ\text{C}$ before sorting; shipping time was 6 h. Pregnancy rates for the sexed sperm from the two bulls were virtually identical, 49% for the unshipped and 52% for the shipped semen. Semen was not diluted with extender and not cooled for shipping because staining properties of sperm with Hoechst 33342 are altered by dilution with extenders. Furthermore, in other studies (see Example 4), storing semen neat at ambient temperature between collection and flow-sorting was found to be superior to diluting it.

Table 16. Results of field trial 7 -- Crossbred beef heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 37
Sexed, frozen/body	1.5×10^6	86	34 (40%) ^a
Sexed, frozen/horns	1.5×10^6	86	53 (62%) ^b
Control, frozen/body	20×10^6	35	18 (51%) ^{a,b}

^{a,b} Means without common superscripts differ ($P < 0.01$).

Field trial 8 (Table 17) concerned feedlot heifers not implanted with growth promotants; at the time pregnancy was diagnosed they were aborted, so calving data was not available. This experiment illustrates that efficacious sexing also can be done in the male direction. Pregnancy rates for the 2 bulls were 50 and 61%.

Table 17. Results of field trial 8 -- Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant ^a day 74 to 76	No. ♂ fetuses
Sexed, frozen, 72 mW laser/body	1×10^6	18	7 (39%)	6 (86%)
Sexed, frozen, 135 mW laser/body	1×10^6	18	13 (78%)	12 (92%)

^a No significant differences.

Field trial 9 (Table 18) was the only trial to show a convincing advantage of 3.0×10^6 sexed, frozen sperm/insemination dose. This advantage was true for both inseminators. Pregnancy rates for sexed sperm from the 2 bulls were 62 and 75%.

5 Table 18. Results of field trial 9 -- Red Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 60 to 63 ^a	No. ♀ fetuses
Sexed, frozen/body	1.5×10^6	15	8 (53%)	7 (88%)
Sexed, frozen/body	3.0×10^6	14	12 (86%)	9 (75%)
Sexed, frozen/horns	1.5×10^6	16	9 (56%)	7 (78%)
Sexed, frozen/horns	3.0×10^6	16	12 (75%)	11 (92%)
Control, frozen/body	20×10^6	30	21 (70%)	13 (62%)

^a 3.0×10^6 sexed sperm had a higher pregnancy rate (80%) than 1.5×10^6 sexed sperm (55%), $P < 0.05$, 1-tail χ^2 .

15 Pregnancy rates in field trial 10 (Table 19) with sexed, frozen semen, were similar to controls; the accuracy of sexing sperm on this trial was only 82%, which, however, is not significantly different from the targeted 90% accuracy. Pregnancy rates for sexed semen were 54, 66, and 50% for bulls AN4, AN7, and AN8, respectively ($P > 0.1$). Eighteen of the heifers
 20 inseminated in this trial were the calves resulting from sexed sperm in field trial 1.

Table 19. Results of field trial 10 -- Angus heifers in Wyoming, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 61 to 63 ^a	No. ♀ fetuses
Sexed, frozen/body	1×10^6	44	26 (59%)	23 (85%)
Sexed, frozen/body	3×10^6	43	23 (53%)	17 (74%)
Control, frozen/body	20×10^6	35	20 (57%)	12 (57%)

^a No significant differences.

Table 20. Results of field trial 11 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 28 to 30 ^a	No. pregnant day 56 to 58 ^{a,b}
5 Sexed, frozen/body	1 x 10 ⁶	12	8 (67%)	7 (58%)
Sexed, frozen/body	3 x 10 ⁶	12	6 (50%)	4 (33%)
Sexed, frozen/horns	1 x 10 ⁶	7	4 (57%)	4 (57%)
Sexed, frozen/horns	3 x 10 ⁶	7	4 (57%)	4 (57%)
Control, frozen/body	20 x 10 ⁶	9	4 (44%)	3 (33%)

^a No significant differences, χ^2 .

^b 16 of 17 (94%) fetuses from the sexed semen treatments were female; 2 were too deep in the body cavity to sex with ultrasound.

Data from trials were combined in which treatments were identical except 1 x 10 and 1.5 x 10⁶ sperm doses were pooled (Table 21).

Table 21. Meta-summary from combining trials with sexed, frozen semen and frozen controls.

Trials combined	Sperm no./site	No. heifers	No. pregnant
5, 6, 9, 11	1.0 - 1.5 x 10 ⁶ /body	77	36 (47%)
	3.0 x 10 ⁶ /body	76	38 (50%)
	1.0 - 1.5 x 10 ⁶ /horns	72	32 (44%)
	3.0 x 10 ⁶ /horns	72	39 (54%)
	20 x 10 ⁶ /body, control	93	61 (66%)
4, 5, 6, 9, 10, 11	1.0 - 1.5 x 10 ⁶ /body	176	98 (56%)
	3.0 x 10 ⁶ /body	171	88 (51%)
	20 x 10 ⁶ /body, control	183	124 (68%)
5, 6, 7, 9, 11	1.5 x 10 ⁶ /body	163	70 (43%)
	1.5 x 10 ⁶ /horn	158	85 (54%)
	20 x 10 ⁶ /body, control	128	79 (62%)

Pregnancy rates with sexed sperm were generally 70-90% of unsexed controls within experiments with 7 to 20 times more sperm. This difference was less in the more recent trials, possibly reflecting improved sexing and sperm-processing procedures.

In some trials, heifers were examined for pregnancy by ultrasound at both 1 and 2 months after insemination. Pregnancy losses in this interval were similar ($P>0.1$) for sexed (23/261; 8.8%) versus control (9/145; 6.2%) sperm treatments, which is one measure that genetic damage due to sexing is minimal. Calving information was obtained from only a few of the pregnant heifers because most cattle from the earlier trials were sold, and those from later trials have not calved yet. The population of calves produced to date from sexed semen appears to be no different from the population of controls.

CONCLUSION

Sex ratios in cattle can be distorted to about 90% of either sex by sorting sperm on the basis of DNA content with a flow cytometer/cell sorter followed by cryopreservation and relatively routine artificial insemination. Calves resulting from sexed sperm appear to be normal. For most bulls in these studies, pregnancy rates with 1.0 to 1.5×10^6 sexed, frozen sperm were 70 to 90% of unsexed controls with 20 or 40×10^6 frozen sperm inseminated conventionally. These results apply to well-managed heifers bred by well-trained inseminators using properly processed semen. There may be a small advantage to inseminating sexed sperm bilaterally into the uterine horns compared to standard uterine body insemination.

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the ends of the present invention.

All patents and publications described are herein incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

- 1 1. A method for the cryopreservation of sperm comprising:
- 2 (a) obtaining a selected sperm sample;
- 3 (b) cooling said selected sperm sample;
- 4 (c) isolating sperm from said selected sperm sample to produce isolated sperm;
- 5 (d) adding final extender to said isolated sperm to produce a suspension of
- 6 sperm; and
- 7 (e) freezing said suspension of sperm.
- 1 2. The method of Claim 1 wherein said selected sperm sample comprises a portion of the
- 2 sperm present in a source sample, said portion of sperm selected for a characteristic, and
- 3 wherein the sperm concentration in the selected sperm sample is lower than in the source
- 4 sample.
- 1 3. The method of Claim 1 wherein said selected sperm sample comprises sex-selected
- 2 sperm.
- 1 4. The method of Claim 1 wherein said selected sperm sample comprises mammalian
- 2 sperm.
- 1 5. The method of Claim 4 wherein said selected sperm sample comprises bovine sperm.
- 1 6. The method of Claim 4 wherein said selected sperm sample comprises equine sperm.
- 1 7. The method of Claim 4 wherein said selected sperm sample comprises porcine sperm.
- 1 8. The method of Claim 1 wherein said selected sperm sample comprises sperm selected by
- 2 a method from the group consisting of flow cytometry, a magnetic technique, a columnar
- 3 technique, a gravimetric technique, a biochemical technique, a technique based on

4 motility of sperm, a technique based on an electrical property of sperm, and any
5 combination thereof.

1 9. The method of Claim 8 wherein said sperm have been selected by flow cytometry.

1 10. The method of Claim 1 wherein cooling is carried out by reducing the temperature of the
2 selected sperm sample to about 5°Celsius.

1 11. The method of Claim 10 wherein cooling is carried out over a period of about 60 minutes
2 to about 240 minutes.

1 12. The method of Claim 1 wherein the final extender added to said selected sperm sample
2 (each comprise, in addition to a cryoprotectant, one or more of the following components:
3 a component that maintains osmolality and buffers pH, an organic substance that reduces
4 cold shock and preserves fertility of sperm, an energy source, a substance that facilitates
5 sperm capacitation, and an antibiotic.

1 13. The method of Claim 12 wherein said cryoprotectant is selected from the group
2 consisting of disaccharides, trisaccharides, and any combination thereof.

1 14. The method of Claim 12 wherein said cryoprotectant is selected from the group
2 consisting of glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any
3 combination thereof.

1 15. The method of Claim 12 wherein said component that maintains osmolality and buffers
2 pH is selected from the group consisting of a buffer comprising a salt, a buffer containing
3 a carbohydrate, and any combination thereof.

1 16. The method of Claim 12 wherein said component that maintains osmolality and buffers
2 pH is selected from the group consisting of sodium citrate,
3 Tris[hydroxymethyl]aminomethane,

4 N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, monosodium glutamate,
5 milk, HEPES buffered medium, and any combination thereof.

1 17. The method of Claim 12 wherein said organic substance is selected from the group
2 consisting of egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin,
3 and any combination thereof.

1 18. The method of Claim 12 wherein said energy source is a monosaccharide selected from
2 the group consisting of glucose, fructose, mannose, and any combination thereof.

1 19. The method of Claim 12 wherein said antibiotic is selected from the group consisting of
2 tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin,
3 and any combination thereof.

1 20. The method of Claim 1 wherein, after the addition of the final extender, the sperm sample
2 and suspension of sperm, respectively, comprise glycerol, sodium citrate,
3 Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

1 21. The method of Claim 1 wherein, after the addition of the final extender, said sperm
2 sample and suspension of sperm, each comprise glycerol, sodium citrate, egg yolk, and
3 one or more antibiotics.

1 22. The method of Claim 1 wherein, after the addition of the final extender, said sperm
2 sample and suspension of sperm, each comprise glycerol, egg yolk, milk, fructose, and
3 one or more antibiotics.

1 23. The method of Claim 1 wherein said extender has a pH in the range of about 6.5 to about
2 7.5.

1 24. The method of Claim 1 wherein the sperm are isolated from said selected sperm sample
2 by centrifugation.

any one of these claims.

See Claim 12.

These don't say daily

So 12 1/2

1 25. The method of Claim 24 wherein said centrifugation allows for at least about 50% to
2 about 90% recovery of sperm.

1 26. The method of Claim 1 wherein the concentration of sperm in said suspension prior to
2 freezing is about $1 \times 10^6/\text{ml}$ to about $300 \times 10^6/\text{ml}$.

1 27. A frozen selected sperm sample comprising a portion of the sperm present in a source
2 sample, said portion of sperm selected for a characteristic.

II 1 28. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 comprises sex-selected sperm.

1 29. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 comprises mammalian sperm.

1 30. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises bovine sperm.

1 31. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises equine sperm.

1 32. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises porcine sperm.

1 33. The frozen selected sperm sample of Claim 27 wherein the method used to select said
2 selected sperm sample comprises a technique from the group consisting of flow
3 cytometry, a magnetic technique, a columnar technique, a gravimetric technique, a
4 biochemical technique, a technique based on motility of sperm, a technique based on an
5 electrical property of sperm, and any combination thereof.

1 34. The frozen selected sperm sample of Claim 33 wherein said frozen selected sperm sample
2 comprises sperm that have been selected by flow cytometry.

1 35. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 is produced by a method comprising:

- 3 (a) obtaining a selected sperm sample;
4 (b) cooling said selected sperm sample;
5 (c) isolating sperm from said selected sperm sample to produce isolated sperm;
6 (d) adding final extender to said isolated sperm to produce a suspension of
7 sperm; and
8 (e) freezing said suspension of sperm.

1 36. A method comprising using the frozen selected sperm sample of Claim 27 for artificial
2 insemination or in vitro fertilization.

1 37. The method of Claim 36 comprising using said frozen selected sperm sample for low-
2 dose artificial insemination.

add
A1

add
B2

ABSTRACT OF THE INVENTION

The present invention provides a method of cryopreserving sperm that have been selected for a specific characteristic. In a preferred embodiment, the method is employed to freeze sex-selected sperm. Although the cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred. The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or bison sperm. The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John L. Schenk
Application No.: Unknown
Filed: December 17, 1999
For: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 C.F.R. §§ 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN**

I hereby declare that I am

- () the owner of the small business concern identified below:
(X) an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: XY, Inc.
ADDRESS: 3801 W. Rampart Road
ARBL Building, CSU Foothills Research Campus
Fort Collins, CO 80523

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 C.F.R. § 121.3-18, for purposes of paying reduced fees under § 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled METHOD OF CRYOPRESERVING SELECTED SPERM CELLS by inventor, John L. Schenk

described in

- (X) the specification filed herewith
() application Serial No. _____, filed _____
() Patent No. [_____], issued [Date].

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

Full Name: _____

Address: _____

() Individual () Small Business Concern () Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 C.F.R. § 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of person signing: DR. MERVYN JACOBSON

Title of person
other than owner: PRESIDENT & CEO.

Address of person signing: XY, Inc.
3801 W. Rampart Road
ARBL Building, CSU Foothills Research Campus
Fort Collins, CO 80523

Signature: [Signature]

Date: DEC 23rd 1999.

DEC 23rd 1999.

¹NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27)

**DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION**

As a below named inventor,

I hereby declare that my residence, post office address and citizenship is as stated below next to my name.

I believe I am the original and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: "METHOD OF CRYOPRESERVING SELECTED SPERM CELLS", the specification of which

 X is attached hereto.

 was filed on and assigned
Application Serial Number

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign applications for patent or inventor's certificate, listed below and so identified, and I have also identified below any foreign application for patent or inventor's certificate on this invention filed by me or my legal representatives or assigns and having a filing date before that of the application on which priority is claimed.

Number	Country	Day/Month/ Year Filed	Priority Claimed - Yes or No

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Application Serial No.	Filing Date	Status

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States provisional application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior provisional application and the filing date of this application.

Provisional Application No.	Filing Date	Status
60/167,423	November 24, 1999	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorneys and agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith and request that all correspondence and telephone calls with respect to this application be directed to McCUTCHEN, DOYLE, BROWN & ENERSEN, LLP Three Embarcadero Center, San Francisco, California 94111, Telephone No. (415) 393-2000:

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Rajiv Yadav	43,999
Vincent K. Yip	42,245

John L. Schenk

John F. Schenk

Date: 21 Dec 99

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Fort Collins, CO 80526

Citizenship:

U. S.

[illegible]

PATENT APPLICATION FEE DETERMINATION RECORD

Effective December 29, 1999

Application or Docket Number

CLAIMS AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA
BASIC FEE		
TOTAL CLAIMS	37 minus 20 = *	17
INDEPENDENT CLAIMS	7 minus 3 = *	
MULTIPLE DEPENDENT CLAIM PRESENT		

* If the difference in column 1 is less than zero, enter "0" in column 2

CLAIMS AS AMENDED - PART II

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT	(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**
Independent	*	Minus	***
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT	(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**
Independent	*	Minus	***
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT	(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**
Independent	*	Minus	***
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

SMALL ENTITY
TYPE ☒

OTHER THAN
SMALL ENTITY

RATE	FEE	OR	RATE	FEE
	345.00	OR		690.00
X\$ 9=	153.12	OR	X\$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL	498.12	OR	TOTAL	

SMALL ENTITY

OTHER THAN
SMALL ENTITY

RATE	ADDI- TIONAL FEE	OR	RATE	ADDI- TIONAL FEE
X\$ 9=		OR	X\$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

RATE	ADDI- TIONAL FEE	OR	RATE	ADDI- TIONAL FEE
X\$ 9=		OR	X\$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

RATE	ADDI- TIONAL FEE	OR	RATE	ADDI- TIONAL FEE
X\$ 9=		OR	X\$18=	
X39=		OR	X78=	
+130=		OR	+260=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

Refund Ref:
01/24/2000 BALEXAND 0000088606

CHECK Refund Total: \$35.00

01/24/2000 BALEXAND 00000016 09478299

CA FC:201
CB FC:203

245.00 CP
135.00 CP

PTO-1556
(5/87)

U.S. GPO 1998-433-214/80404



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.

09/478,299 01/05/00 Schenk

J 22091-701

EXAMINER

HM22/0412

Emily M. Haliday
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Three Embarcadero Center
San Francisco CA 94111-4066

MELLER, M

ART UNIT

PAPER NUMBER

1651

DATE MAILED:

04/12/00

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Office Action SummaryApplication No.
09/478,299Applicant(s)
SchenkExaminer
Michael MellerGroup Art Unit
1651☒ Responsive to communication(s) filed on Jan 5, 2000☐ This action is **FINAL**.☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire one month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims☒ Claim(s) 1-37 is/are pending in the application.

Of the above, claim(s) _____ is/are withdrawn from consideration.

☐ Claim(s) _____ is/are allowed.☐ Claim(s) _____ is/are rejected.☐ Claim(s) _____ is/are objected to.☒ Claims 1-37 are subject to restriction or election requirement.**Application Papers**☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.☐ The drawing(s) filed on _____ is/are objected to by the Examiner.☐ The proposed drawing correction, filed on _____ is ☐ approved ☐ disapproved.☐ The specification is objected to by the Examiner.☐ The oath or declaration is objected to by the Examiner.**Priority under 35 U.S.C. § 119**☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been
☐ received.☐ received in Application No. (Series Code/Serial Number) _____☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____

☒ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).**Attachment(s)**☐ Notice of References Cited, PTO-892☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____☐ Interview Summary, PTO-413☐ Notice of Draftsperson's Patent Drawing Review, PTO-948☐ Notice of Informal Patent Application, PTO-152

--- SEE OFFICE ACTION ON THE FOLLOWING PAGES ---

Art Unit: 1651

DETAILED ACTION

Election/Restriction

1. Restriction to one of the following inventions is required under 35 U.S.C. 121:
 - I. Claims 1-26 and 35, drawn to a method of making a product and a product by process, classified in class 435, subclass various.
 - II. Claims 27-34, drawn to a product, classified in class 435, subclass 2.
 - III. Claims 36 and 37, drawn to a method of using the product, classified in class 424, subclass 520.
2. The inventions are distinct, each from the other because of the following reasons:

Inventions I and II are related as process of making and product made. The inventions are distinct if either or both of the following can be shown: (1) that the process as claimed can be used to make other and materially different product or (2) that the product as claimed can be made by another and materially different process (MPEP § 806.05(f)). In the instant case the product can be made by using a different final extender such as water or a salt.
3. Inventions^{I-II}_I and^{III}_I are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product (MPEP § 806.05(h)). In the instant case the product can be used as a substrate for an enzyme in an assay.

Art Unit: 1651

4. This application contains claims directed to the following patentably distinct species of the claimed invention: the species of claim 12, specifically what cryoprotectant and what additional component.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claim 1 is generic.

Applicant is advised that a reply to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 CFR 1.141. If claims are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a).

Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

Art Unit: 1651

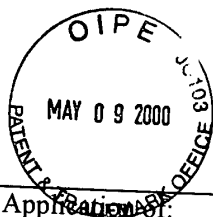
5. A telephone call was made to Emily Haliday on 4/10/2000 to request an oral election to the above restriction requirement, but did not result in an election being made.

Applicant is advised that the reply to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed (37 CFR 1.143).

Any inquiries concerning this communication should be directed to Examiner Mike Meller at telephone number (703) 308-4230. If attempts to reach the examiner by telephone are unsuccessful, the Examiner's supervisor, Michael Wityshyn, can be reached at (703) 308-4743. The Fax phone number for the art unit is (703) 308- 0294. Any inquiries of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.



Christopher Tate
Patent Examiner



In The United States Patent and Trademark Office

In re Application of:

Assignee: XY, Inc.

Inventors: John L. Schenk

Application No.: 09/478,299

Filed: January 5, 2000

For: METHOD OF
CRYOPRESERVING
SELECTED SPERM
CELLS

Group Art Unit: 1648

Examiner: Not yet assigned

Attorney Docket No.: 22091-701 CON1

Certificate of Mailing Under 37 C.F.R. § 1.8

Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Date: May 4, 2000

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Type or Print Name of Person Mailing: Laurie Oppenheimer

MAY 15 2000

Laurie Oppenheimer
Signature of Person Mailing

TECH CENTER 1600/2900

644 1648
#3
TDS
w/attach
5/15/00

INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In accordance with 37 C.F.R. § 1.56, the references listed below and on the attached Form PTO-1449 are being brought to the attention of the Examiner for consideration in connection with the examination of the above-identified patent application.

1. United States Patent No. 4,267,268, Issued May 12, 1981;
2. United States Patent No. 4,980,277, Issued December 25, 1990;
3. United States Patent No. 5,135,759, Issued August 4, 1992;
4. United States Patent No. 5,514,537, Issued May 7, 1996;
5. International Publication No. WO 99/33956, Publication Date July 8, 1999;

6. Amann, Rupert P., "Cryopreservation of Sperm," *Encyclopedia of Reproduction*, 1:773-783 (1999);
7. Arriola, J. and Foote, R. H., "Glycerolation and Thawing Effects on Bull Spermatozoa Frozen in Detergent-Treated Egg Yolk and Whole Egg Extenders," *J Dairy Sci*, 70:1664-1670 (1987);
8. Behrman, S. J. and Ackerman, D. R., "Freeze Preservation of Human Sperm," *Am. J. Obstet. and Gynecol.*, 103:5:654-664 (1969);
9. Foote, R. H., "Buffers and Extenders: What Do They Do? Why Are They Important?" *Proc of the 10th NAAB Tech. Conf. on Artificial Insemination and Reproduction*, 62-70 (1984);
10. Graham, J. K. and Hammerstedt, R. H., "Differential Effects of Butylated Hydroxytoluene Analogs on Bull Sperm Subjected to Cold-Induced Membrane Stress," *Cryobiology*, 29:106-117 (1992);
11. Hammerstedt, et al, "Cryopreservation of Mammalian Sperm: What We Ask Them to Survive," *Journal of Andrology*, 11:1:73-88 (1990);
12. Maxwell, W.M.C., and Johnson, L. A., "Chlortetracycline Analysis of Boar Spermatozoa After Incubation, Flow Cytometric Sorting, Cooling, or Cryopreservation," *Molecular Reproduction and Development*, 46:408-418 (1997);
13. Polge, et al, "Revival of Spermatozoa After Vitrification and Dehydration at Low Temperatures," *Nature*, 164:666 (1949);
14. Pursel, et al, "Effect of Orvus ES Paste on Acrosome Morphology, Motility and Fertilizing Capacity of Frozen-Thawed Boar Sperm," *Journal of Animal Science*, 47:1:198-202 (1978);
15. Salisbury, et al, *Physiology of Reproduction and Artificial Insemination of Cattle*, 2nd Ed., San Francisco: W. H. Freeman, 442-554 (1978);
16. Schenk, et al, "Cryopreservation of Flow-Sorted Bovine Spermatozoa," *Theriogenology*, 52:1375-1391 (1999);

Information Disclosure Statement -- Section 9 PTO-1449 (Modified) [6-1] Page 2 of 6

52018387.1

Docket No.: 22091-701 CON1

17. Seidel, et al, "Insemination of Heifers with Sexed Sperm," *Theriogenology*, 52:1407-1420 (1999);
18. Watson, P. F., "Recent Developments and Concepts in the Cryopreservation of Spermatozoa and the Assessment of Their Post-Thawing Function," *Reprod. Fertil. Dev.*, 7:871-891 (1995).

I. Timing of the Information Disclosure Statement:

This Information Disclosure Statement:

- ☐ Accompanies the new patent application submitted herewith. 37 CFR § 1.97(a).
- ☐ Is filed within three months after the filing date of the application or within three months after the date of entry of the national stage of a PCT application as set forth in 37 CFR § 1.491.
- ☒ As far as is known to the undersigned, is filed before the mailing date of a first Office Action on the merits. In the event, however, that an Office Action has crossed in the mail with this Information Disclosure Statement, the Commissioner is hereby authorized to charge Deposit Account No. 13-0257 for any fees required pursuant to 37 CFR §§ 1.17(p) or 1.17(i)(1).
- ☐ Is filed after the first Office Action and more than three months after the application's filing date or PCT national stage date of entry filing but, as far as is known to the undersigned, prior to the mailing date of either a final rejection or a notice of allowance and is accompanied by either the fee (\$240) set forth in 37 CFR § 1.17(p) or a certification as specified in 37 CFR § 1.97(e), as checked below.
- ☐ Is filed after the mailing date of either a final rejection or a notice of allowance, whichever occurred first, and is accompanied by the fee (\$130) set forth in 37 CFR § 1.17(i)(1) and a certification as specified in 37 CFR § 1.97(e), as checked below. This document is to be considered as a petition requesting consideration of the Information Disclosure statement.

Information Disclosure Statement -- Section 9 PTO-1449 (Modified) [6-1] Page 3 of 6

52018387.1

Docket No.: 22091-701 CON1

The undersigned certifies that:

☐ Each item of information contained in the Information Disclosure Statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure Statement.

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MAY 15 2008

TECH CENTER 1600/2900

☐ No item of information contained in this Information Disclosure Statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application or, to the knowledge of the undersigned after making reasonable inquiry, was known to any individual designated in 37 CFR § 1.56(c) more than three months prior to the filing of this Information Disclosure Statement.

II. Copies of the Cited Items:

☒ Copies of all of the items listed on the attached Form PTO-1449 are enclosed.

☐ Copies of only the following items listed on the attached Form PTO-1449 are enclosed:

☐ Copies of those items which are marked with an asterisk (*) in the attached Form PTO-1449 are not supplied because they were previously cited by or submitted to the Office in a prior Application No. _____, filed _____, and relied upon in this application for an earlier filing date under 35 U.S.C. § 120. See 37 C.F.R. § 1.98(d).

☐ Copies of those items which are marked with a double asterisk (**) in the attached Form PTO-1449 are not supplied because they are U.S. patent application(s) and no copy of a U.S. patent application needs to be provided pursuant to 37 C.F.R. § 1.98(a)(2)(iii).

III. Concise Explanation of Relevance:

☐ A concise explanation of relevance of the items listed on Form PTO-1449 is not given.

☐ A concise explanation of relevance of [some of] the items listed on Form PTO-1449 is in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references [copy attached].

☐ A concise explanation of relevance of the items listed on form PTO-1449 is given for only non-English language listed item(s). Specifically, the following foreign-language documents are being brought to the attention of the Examiner along with a concise explanation of the relevance of each document, as it is presently understood by the individual designated in 37 C.F.R. § 1.56(c) most knowledgeable about the content of the information in each document:

IV. Conclusion:

Citation of the above documents shall not be construed as:

1. an admission that the documents are necessarily prior art with respect to the instant invention;
2. a representation that a search has been made, other than as described above; or
3. an admission that the information cited herein is, or is considered to be, material to patentability as defined in § 1.56(b).

It is respectfully requested that the Examiner indicate consideration of the cited references by returning a copy of the attached form PTO 1449 with initials or other appropriate marks.

Serial No.: 09/478,299

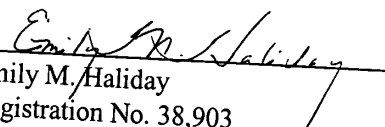
The Commissioner is hereby authorized to charge Deposit Account No. 50-1189 for any additional fees required in connection with the filing of this Information Disclosure Statement.

Please address all correspondence regarding this communication to the following address:

Emily M. Haliday
McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (650) 849-4910

Dated: May 4, 2000

Respectfully submitted,

By: 
Emily M. Haliday
Registration No. 38,903

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (650) 849-4910
Telefax: (650) 849-4800

Information Disclosure Statement -- Section 9 PTO-1449 (Modified) [6-1] Page 6 of 6

S2018387.1
Docket No.: 22091-701 CON1



In The United States Patent and Trademark Office

#4
Elee
5/24/00

In re Application of:

Assignee: XY, Inc.

Inventors: John Schenk

Serial No: 09/478,299

Filed: January 5, 2000

For: METHOD OF
CRYOPRESERVING
SELECTED SPERM CELLS

Group Art Unit: 1648

Examiner: Not yet assigned

Attorney Docket No.: 22091-701 CON1

Certificate of Mailing Under 37 C.F.R. § 1.8

Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Date: May 12, 2000

Type or Print Name of Person Mailing: Laurie Oppenheimer

Laurie Oppenheimer
Signature of Person Mailing

RESPONSE TO RESTRICTION REQUIREMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

This paper is submitted in response to the Office Action dated April 12, 2000 in the above-identified patent application.

REMARKS

Claims 1-37 are pending in the application.

Restriction Requirement

In the Office Action, the Examiner identified three claim groups and required the election of one claim group for prosecution in the present application. The three claim groups are as follows:

- I. Claims 1-26 and 35, "drawn to a method of making a product and a product by process;"
- II. Claims 27-34, "drawn to a product;"
- III. Claims 36 and 37, "drawn to a method of using the product;"

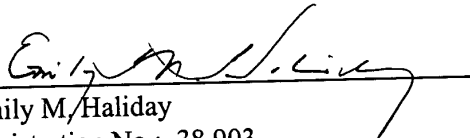
Applicant hereby elects Claim Group I for prosecution in this application.

Conclusion

If the Examiner has any questions regarding this paper, the Examiner is invited to call the undersigned.

Dated: May 12, 2000

Respectfully submitted,

By: 

Emily M. Haliday

Registration No.: 38,903

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (650) 849-4910
Telefax: (650) 849-4800



GP1648

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Assignee: XY, Inc.

Inventors: John Schenk

Serial No: 09/478,299

Filed: January 5, 2000

For: METHOD OF
CRYOPRESERVING
SELECTED SPERM CELLS

Group Art Unit: 1648

Examiner: Not yet assigned

Attorney Docket No.: 22091-701 CON1

Certificate of Mailing Under 37 C.F.R. § 1.8

Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Date: May 12, 2000

Type or Print Name of Person Mailing: Laurie Oppenheimer

Laurie Oppenheimer

Signature of Person Mailing

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

RESPONSE & FEE TRANSMITTAL

RECEIVED
MAY 17 2000
TC 1500 MAIL ROOM

Sir:

In response to the Office Action mailed on April 12, 2000, enclosed herewith for filing are the following.

- ☒ A Response to the Office Action (Restriction Requirement)
☐ Revocation of Power of Attorney and Appointment of New Attorney
☐ Amendment after Final Action Under 37 C.F.R. § 1.116
☐ Request for Extension of Time (one month) to File Response Under 37 C.F.R. § 1.136 (a).
☐ A Check in the amount of _____ for the fee for the Extension of Time is enclosed:
☐ Charge the fee of _____ for the Extension of Time to Deposit Account No. 50-1189.
☐ Docket No.: _____. A DUPLICATE COPY OF THIS SHEET IS ATTACHED.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required in this application under 37 C.F.R. Sections 1.16-1.17 or to credit any overpayment, to Deposit Account No. 50-1189, Docket No.: 22091-701 CON1. Should no proper payment be enclosed herewith, as by a check being in the wrong account, unsigned, post-date, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 50-1189, Docket No.: 22091-701 CON1. A DUPLICATE COPY OF THIS SHEET IS ATTACHED.



Fee Calculation Claims

					SMALL ENTITY		OTHER	
					Rate	Fee	Rate	Fee
Basic Fee	Current Claims		Highest Previous					
Total Claims	37	-	37	= 0	x 9	\$0	x 18	\$
Indep. Claims	3	-	3	= 0	x 39	\$0	x 78	\$
Multiple Dependent Claim(s):					+ 130	\$	+ 260	\$
					TOTAL:	\$0	TOTAL:	\$

Please address all correspondence regarding this communication to the following address:

Emily M. Haliday
 McCutchen, Doyle, Brown & Enersen, LLP
 Three Embarcadero Center
 San Francisco, California 94111
 (650) 849-4910

Respectfully submitted,

Dated: May 12, 2000

By: Emily M. Haliday
 Emily M. Haliday
 Registration No.: 38,903

Mailing Address:

McCutchen, Doyle, Brown & Enersen, LLP
 Three Embarcadero Center
 San Francisco, California 94111
 Telephone: (415) 393-2000
 Telefax: (415) 393-2286



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
09/478,299	01/05/00	SCHENK	J 22091-701

Emily M. Haliday
McCutchen Doyle Brown & Enersen LLP
Three Embarcadero Center
San Francisco CA 94111-4066

HM22/0526

EXAMINER

ART UNIT	PAPER NUMBER

1651
DATE MAILED:

05/26/00

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

The timely submission under 37 CFR 1.129(a) filed on 5/15/2000 is not fully responsive to the prior Office action because it did not address the election of species as set forth on page 3, lines 1-3 of the Office Action mailed 4/12/2000. Since the submission appears to be a *bona fide* attempt to provide a complete reply to the prior Office action, applicant is given a shortened statutory period of ONE MONTH or THIRTY DAYS from the mailing date of this letter, whichever is longer, to submit a complete reply. This shortened statutory period supersedes the time period set in the prior Office action. This time period may be extended pursuant to 37 CFR 1.136(a). If a notice of appeal and the fee set forth in 37 CFR 1.17(e) were filed prior to or with the payment of the fee set forth in 37 CFR 1.17(r), the payment of the fee set forth in 37 CFR 1.17(r) by applicant is construed as a request to dismiss the appeal and to continue prosecution under 37 CFR 1.129(a). The appeal stands dismissed.

Any inquiries concerning this communication should be directed to Examiner Mike Meller at telephone number (703) 308-4230. If attempts to reach the examiner by telephone are unsuccessful, the Examiner's supervisor, Michael Wityshyn, can be reached at (703) 308-4743. The Fax phone number for the art unit is (703) 308- 0294. Any inquiries of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.



Christopher Tate
Patent Examiner

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a))

I hereby certify that this correspondence is, on the date shown below, being:

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FACSIMILE

[] transmitted by facsimile to the Patent and Trademark Office.

Name of Person Certifying: Carol M. Gruppi

Date: October 26, 2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): John Schenk

Serial No.: 09/478,299

Filing Date: January 5, 2000

Title: Method of Cryopreserving Selected Sperm Cells

Assignee: XY, Inc.

Examiner: M. Meller

Group Art Unit: 1651

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ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

RESPONSE TO RESTRICTION REQUIREMENT UNDER 35 U.S.C. § 121

Dear Sir:

This is in response to the Office Action dated May 26, 2000. This response is accompanied by a petition for an extension of time to extend the period to respond to the Office Action to October 26, 2000 and the appropriate fee under 37 C.F.R. § 1.16 and § 1.17. Accordingly this response is timely filed.

The Office Action dated May 26, 2000, requested that Applicant elect a species for prosecution on the merits as requested in the Restriction Requirement dated April 12, 2000. In the Restriction Requirement dated April 12, 2000, the Examiner stated that there were distinct inventions and required Applicants to elect one invention for prosecution on the merits. The distinct inventions, as set forth in the Restriction Requirement, are as follows:

I. Claims 1-26 and 35, drawn to methods of making a product and a product by process, classified in class 435, subclass various.

II. Claims 27-34, drawn to a product, classified in class 435, subclass 2; and

III. Claims 36 and 37, drawn to a method of the using the product, classified in class 424, subclass 520.

The Restriction Requirement also stated that the Claims were directed to patentably distinct species. Specifically, election of a species for the “cryoprotectant” and “additional component” recited in Claim 12 for prosecution on the merits was requested. The Examiner stated that Claim 1 was generic.

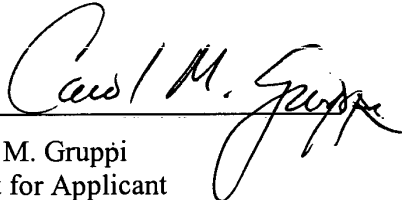
In a response filed on April 12, 2000, Applicant elected the Claims of Group I for prosecution on the merits but did not elect a species for the “cryoprotectant” and “additional component” recited in Claim 12. Applicant provisionally elects glycerol (Claim 14) as a species for the cryoprotectant. As a species for the additional component, Applicant provisionally elects a component that maintains osmolality and buffers pH (Claim 12), such as a buffer comprising a salt (Claim 15). The claims readable on these species are 1-26 and 35. Applicants, however, traverse the the election of species for the reasons presented below.

A Restriction Requirement requires the inventions be independent or distinct as claimed, and there must be a serious burden on the Examiner for restriction to be required (*see* MPEP § 803). Ten independent and distinct nucleotide sequences have been determined to be a “reasonable number” of sequences to be claimed and examined in a single application without restriction (*see* MPEP § 803.04). Thus, examination of ten independent and distinct nucleotide sequences has been determined not constitute an undue burden for the Examiner (*see* MPEP § 803.04).

Likewise, Applicants submit that it would not be unduly burdensome for the Examiner to search for more than one additional component or more than one cryoprotectant. Accordingly, reconsideration of the election of species is respectfully requested. If Examiner has any questions concerning this Response, the Examiner is respectfully requested to telephone Applicant’s agent at the telephone number given below.

A fee of \$695.00 is due under 37 C.F.R. § 1.16 and § 1.17 for the Petition for a
an Extension of Time filed concurrently herewith. The Assistant Commissioner is hereby
authorized to charge any additional fees which may be required by this paper, or credit any
overpayment to Deposit Account No. 50-1189. Docket No.: 22091-701CON1. A DUPLICATE
COPY OF THIS SHEET IS ATTACHED.

Respectfully submitted,

By: 
Carol M. Gruppi
Agent for Applicant
Registration No.: 37,341

Dated: October 26, 2000

Mailing Address:

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (650) 849-4400
Telefax: (650) 849-4800

849-4902

1651
#1
11.11.00
Docket No.: 22091-701 CON1

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a))

I hereby certify that this correspondence is, on the date shown below, being:

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[x] deposited with the United States Postal Service as First Class Mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.
FACSIMILE
[] transmitted by facsimile to the Patent and Trademark Office.

Date: October 26, 2000

Name of Person Certifying: Carol M. Grupp

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): John Schenk

Assignee: XY, Inc.

Serial No.: 09/478,299

Examiner: M. Meller

Filing Date: January 5, 2000

Group Art Unit: 1651

Title: Method of Cryopreserving Selected Sperm Cells

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Assistant Commissioner for Patents
Washington, D.C. 20231

RESPONSE & FEE TRANSMITTAL

Sir:

In response to the Restriction Requirement mailed on May 26, 2000, enclosed herewith for filing are the following:

- ☐ A Response/Amendment [] page(s)
- ☒ A Response to Restriction Requirement under 35 USC 121 [3] page(s)
- ☐ An Amendment Under 37 CFR § 1.111 [] page(s)
- ☐ An Amendment Under 37 CFR § 1.116 [] page(s)
- ☐ Other _____ [] page(s)

Also included are:

- ☒ A Petition for Extension of Time [4] months [2] page(s)
- ☐ Information Disclosure Statement
[] page(s) of PTO-1449 [] copies of IDS citations
- ☒ Verified Statement of Small Entity Status under 37 CFR § 1.27
☐ attached hereto ☒ was previously filed
- ☐ Other: _____

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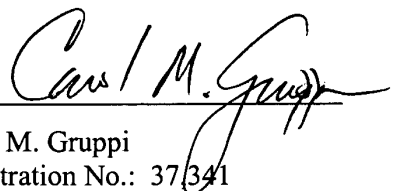
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☒ Return Postcard

Fee Calculation						TECH CENTER 1600/2900
☒ The following fees are submitted:						CALCULATIONS
EXTRA CLAIMS FEE				OTHER THAN SMALL ENTITY	SMALL ENTITY	\$
CLAIMS	CURRENT #	# OF CLAIMS PREVIOUSLY PAID	# EXTRA	RATE	RATE	
Total Claims	37-	37		× \$18.00	× \$9.00	\$0.0
Independent claims	3-	3		× \$80.00	× \$40.00	\$0.0
MULTIPLE DEPENDENT CLAIM(S)						
☐ Yes ☐ No				\$270.00	\$135.00	\$
Petition for Extension of Time Fee (4 months)						\$695.00
OTHER FEES _____ (specify)						\$
TOTAL FEES =						\$695.00

- ☒ Conditional Petition for Extension of Time: An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701CON1 in the amount of \$695.00 to cover the above-fees. *A duplicate copy of this sheet is enclosed.*
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701CON1. *A duplicate copy of this sheet is enclosed.*

Respectfully submitted,

By: Carol M. Gruppi
Registration No.: 37341Date: October 26, 2000Mailing Address:

McCutchen, Doyle, Brown & Enersen, LLP
 Three Embarcadero Center
 San Francisco, California 94111
 Telephone: (650) 849-4400
 Telefax: (650) 849-4800

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a))

I hereby certify that this correspondence is, on the date shown below, being:

MAILING
☒ deposited with the United States Postal Service as First Class Mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.
 Date: October 26, 2000

FACSIMILE
☐ transmitted by facsimile to the Patent and Trademark Office.
 Name of Person Certifying: Carol M. Gruppi

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): John Schenk

Serial No.: 09/478,299

Filing Date: January 5, 2000

Title: Method of Cryopreserving Selected Sperm Cells

Assignee: XY, Inc.

Examiner: M. Meller

Group Art Unit: 1651

Assistant Commissioner for Patents
 Washington, D.C. 20231

PETITION AND FEE FOR EXTENSION OF TIME
 (37 C.F.R. § 1.136(a))

Sir:

Pursuant to 37 C.F.R. § 1.136(a), Applicant hereby petitions for a four month extension of time to respond to the Office Action mailed on May 26, 2000.

1. This communication is in connection with the matter for which this extension is requested

- a. ☒ is filed herewith; or
 b. ☐ has been filed on _____.

2. Applicant is a Small Entity - A verified statement is claiming small entity status

- a. ☐ is attached; or
 b. ☒ has already been filed; or
 c. ☐ does not apply.

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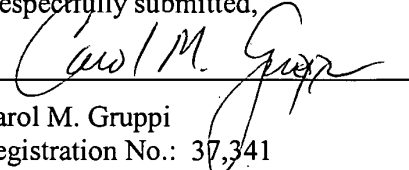
695.00 CH

3. The following fees are submitted:

	TOTAL MONTHS REQUESTED	OTHER THAN SMALL ENTITY	SMALL ENTITY	CALCULATIONS
a. ☐	one month	\$110.00	\$55.00	\$
b. ☐	two month	\$390.00	\$195.00	\$
c. ☐	three month	\$890.00	\$445.00	\$
d. ☒	four month	\$1,390.00	\$695.00	\$695.00
e. ☐	five month	\$1,890.00	\$945.00	\$
f. ☐	An extension for ____ months has already been secured for filing the above-identified communication and the fee paid therefor of \$____ is deducted from the total fee due for the total months of extension now requested. The fee for this extension (\$____), minus the fee previously paid (\$____) equals \$____ (total fee due).			\$
TOTAL FEES =				\$695.00

- ☒ **Conditional Petition for Extension of Time:** An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$_____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701CON1, in the amount of **\$695.00** to cover the above-fees. *A duplicate copy of this sheet is enclosed.*
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701CON1. *A duplicate copy of this sheet is enclosed.*

Dated: October 26, 2000

Respectfully submitted,
 By: 
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USPT,JPAB,EPAB,DWPI,TDBD	l7 same l8	76	<u>L10</u>
USPT,JPAB,EPAB,DWPI,TDBD	l7 and l8	233	<u>L9</u>
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USPT,JPAB,EPAB,DWPI,TDBD	sperm\$5	15549	<u>L7</u>
USPT	(flow cytometry) and l3	20	<u>L6</u>
USPT	(flow cytometry) and l3	20	<u>L5</u>
USPT	flow cytometry and l3	20	<u>L4</u>
USPT	glycerol and l2	143	<u>L3</u>
USPT	centrifug\$5 and l1	630	<u>L2</u>
USPT	((435/2)!.CCLS.)	952	<u>L1</u>

WEST

Generate Collection

L6: Entry 19 of 20

File: USPT

Jun 4, 1991

DOCUMENT-IDENTIFIER: US 5021244 A

TITLE: Sex-associated membrane antibodies and their use for increasing the probability that offspring will be of a desired sex

BSPR:

Another potential separation approach for X-sperm and Y-sperm is based on the known differences in the DNA contents of X-sperm and Y-sperm. Because the DNA content of X-sperm cells is greater than the DNA content of Y-sperm cells, investigators hoped that the respective live cell populations could be separated by density gradient sedimentation or flow cytometry. However, neither has proven to be possible.

BSPR:

The present invention also provides for a method to separate living cells based on DNA content. The method involves separating the cells by flow cytometry, the cytometer having been improved to substantially increase its ability to recognize fine distinctions in fluorescence. Sperm cell populations separated in this manner result in subpopulations enriched in X- and Y-sperm. By isolating the membranes from these enriched subpopulations, one obtains another aspect of this invention: X- and Y-enriched sperm-plasma-membrane vesicles.

DEPR:

Immediately after collection we diluted freshly ejaculated mammalian semen with isoosmotic PBS to 15 ml, and slowly cooled it to 5.degree. C. At this temperature, we washed the sperm three times in isoosmotic, pH 7.2, tris-methylaminomethane buffered saline (TBS) saturated with phenylmethylsulfonylfluoride (PMSF) and 10 .mu.g/ml Hoechst No. 33342 bis benzimide dye. This solution stained cells and simultaneously inhibited enzymatic breakdown. We centrifuged the cells at 483.times.g for 20 minutes and resuspended them to remove seminal plasma proteins. We then diluted the washed cells in dye solution to a cell concentration of 20.times.10.sup.6 cells/ml and allowed them to stain for a minimum of 2 hours. (See Arndt-Jovin et al., 1977.) The Hoechst dye is available from Calbiochem-Behring Corp., San Diego, California. We prepared sheath fluid in the same manner as the solution used to wash sperm, except that the dye was omitted. We degassed the fluid as described above. The objective was to match the refractive indices between sheath and sample fluid.

DEPR:

We cavitated cell samples containing enriched X-sperm or Y-sperm subpopulations in Parr bombs. Suitable sample sizes were 3 to 10 ml with 50,000 to 500,000 cells/ml. We also cavitated non-enriched sperm samples (50:50 mixture of X- and Y-sperm). We used a cavitation method (at about 650 psi) as described by Gillis, et al., 1978. We separated plasma membrane vesicles consisting of mostly (e.g., 80% (boar sperm data)) head plasma membrane and some (e.g., 20% (boar sperm data)) tail plasma membrane from sperm heads, tails, and other particulates by pelleting centrifugation twice at 2500 x g for 30 minutes. We withdrew the supernatant containing the PMV material and centrifuged it at 100,000 x g to obtain the PMV material, which we resuspended and washed in 10 mM tris acetate (pH 5.5). This removed most of the TBS/PMSF/stain from the isolated PMV. Using this procedure we obtained three plasma membrane vesicles populations: (1) X-enriched sperm plasma

membrane vesicles (PMV-X) (approximately 68% X-sperm); (2) Y-enriched plasma membrane vesicles (PMV-Y) (approximately 72% Y-sperm); and (3) non-enriched plasma membrane vesicles (PMV-X/Y) (approximately equal amounts of X- and Y-sperm). The pelleted material resulting after centrifugation of X- and Y-enriched sperm subpopulations is the X- and Y-enriched non-membrane sperm component.

DEPR:

In a preferred 2-D gel separation process of this invention, we began with solubilized plasma membrane vesicles from whole sperm and from X- and Y-enriched sperm subpopulations isolated as in Example II. The LKB 2117 Multiphor II Electrophoresis System laboratory manual gives instructions and formulations for pouring 5.0% T, 2.7% C, 0.5 mm thick IPG gels with a broad range pH gradient of 4-10. We allowed gel polymerization to proceed for 1 hour in an oven heated to 50.degree. C. Following polymerization, we removed gels from the mold, washed them twice for 30 minutes in HPLC water and then rinsed them for 30 minutes in a solution of 2.5% glycerol. We air-dried the gels overnight in a dust free cabinet. Prior to focusing, we rehydrated IPG gels with a solution consisting of 8M urea, 10 mM dithiothreitol (DTT), 0.5% (volume/volume) nonidet p-40 (NP-40), 0.5% carrier ampholyte (CA). We utilized different brands of carrier ampholyte (LKB Ampholine.RTM., Pharmalyte.RTM., and Servalyte.RTM. 3-10) to insure the best possible pH distribution. It is important that the carrier ampholyte used span the entire pI range over which the sample is to be focused. We included carrier ampholyte in rehydration of the gel in order to decrease hydrophobic interaction with basic immobilines.

DEPR:

We solubilized bull plasma membrane proteins with a solution containing 9M urea, 2% (weight/volume) DTT 2% (volume/volume) NP-40, 0.8% (volume/volume) carrier ampholyte. To aid in solubilization, we sonicated samples in a water bath sonicator at 4.degree. C. for 10 minutes. We pelleted remaining aggregates by centrifugation at 13,000.times.g for 10 minutes.

DEPR:

Following isoelectric focusing, we cut the gel into strips corresponding with sample wells. We incubated the strips in equilibration buffer and loaded them directly onto SDS slab gels. We equilibrated for 30 minutes with gentle shaking at room temperature in 8 ml of a solution containing 0.05M tris-HCl pH 6.8, 6M urea, 2% (weight/volume) SDS, 1% (weight/volume) DTT, 30% glycerol and 0.001% bromphenol blue. Following equilibration we rinsed the gel strips briefly to remove excess equilibration buffer and loaded them directly onto vertical 11.0% T, 2.7% C, 1.5 mm thick SDS slab gels with a stacker of 4.8% T, 2.7% C. We electrophoresed proteins at 100 volts until the dye front moved through the stacker. We then increased the voltage to 140 volts for the remainder of the run.

DEPR:

One day prior to fusion, myeloma cells were split to ensure that they were in log phase growth. On the day of fusion, the animal was sacrificed and splenectomized. We rinsed the spleen in DMEM or RPMI-1640 (we prefer the latter) and teased it to separate splenocytes. (we now separate using syringe and needle perfusion.) Typical cell recovery from 1 spleen was 1.times.10.sup.8 cells. We washed splenocytes 3 times in DMEM or RPMI 1640. We prefer the latter.) The myeloma cells were also washed 3 times. We counted splenocytes and myeloma cells and mixed them together at a ratio of 7 spleen cells:1 myeloma cell. (We currently use 1:1.) We then centrifuged the cells at 1000 rpm (Mistral 3000) and decanted the supernatant.

DEPR:

Fusion was done with 1 ml of PEG for a period of one minute with gentle agitation. (We currently adjust PEG to pH 7.) We stopped the reaction with 20 ml DMEM (or preferably RPMI 1640) and centrifuged the mixture as before. We decanted the DMEM (or preferably RPMI 1640) and gently resuspended the pellet of fused cells in 12 ml of HAT (hypoxanthine aminopterin thymidine) medium and plated the cells into twenty-four wells (1 ml of cell suspension/well).

(We currently use HMT--hypoxanthine methotrexate thymidine.) We incubated plates overnight in a 7% CO₂ incubator at 37.degree. C. The next day we fed the wells an additional ml of HAT (or HMT) medium and left them to incubate for 7 to 14 days. We removed the HAT (HMT) medium and replaced it with 10% FCS/DMEM or RPMI. (We prefer the latter.) We incubated plates until colony formation was visible. After the colonies had expanded into larger cultures, we froze them at -70.degree. C. and stored them long-term in liquid nitrogen.

DEPR:

We bound monoclonal antibodies to whole sperm by the following preferred method: We thawed antibody supernatants in a 37.degree. C. bath and diluted with a combination of Dulbecco's/PBS, Hoechst stain, and BSA as previously described. We diluted a whole sperm sample with a labeling solution to a final concentration of 480.times.10.sup.6 sperm cells per 12 mls. We added monoclonal antibody (ca. 200 .mu.l supernatant) to 1.times.10.sup.6 cells and allowed it to incubate for 1 hour at room temperature. We centrifuged the cells at 100.times.g for 5 minutes, discarded the supernatant and resuspended the cell pellet in 200 .mu.l of a 1:20 dilution of an affinity-purified anti-mouse antibody conjugated with R-Phycoerythrin. Following a second centrifugation, we discarded the supernatant and resuspended the cell mixture in a 10% neutral buffered formalin solution.

DEPC:

Sorting Sperm by Flow Cytometry

DEPU:

D. L. Garner et al., "Quantification Of The X-and Y-Chromosome-Bearing Spermatozoa of Domestic Animals By Flow Cytometry", Biol. Repro., 28, pp. 312-21 (1983) ("Garner-I")

DEPU:

J. M. Morrell et al., "Sexing of sperm by Flow Cytometry", Vet. Record., 122, pp. 322-324 (1988)

CCXR:

435/2

ORPL:

D. L. Garner et al., "Quantification Of The X-and Y-Chromosome-Bearing Spermatozoa of Domestic Animals By Flow Cytometry", Biol. Repro., 28, pp. 312-321 (1983) (Garner-I).

ORPL:

J. M. Morrell et al., "Sexing of Sperm by Flow Cytometry", Vet. Record., 122, pp. 322-324 (1988).

WEST

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L6: Entry 2 of 20

File: USPT

Jun 6, 2000

DOCUMENT-IDENTIFIER: US 6071689 A

TITLE: System for improving yield of sexed embryos in mammals

BSPR:

This invention relates generally to the field of sex selection in mammalian offspring. It is especially relevant to the aspect of low dose artificial insemination for creating the desired sex of offspring. Particularly, the invention relates to systems for sorting sperm via flow cytometry for sex-specific and low dose efforts at artificial insemination or the like.

BSPR:

At present, the only quantitative technique used to achieve the separation of X-and Y-chromosome bearing sperm has been that involving individual discrimination and separation of the sperm through the techniques of flow cytometry. This technique appeared possible as a result of advances and discoveries involving the differential dye absorption of X-and Y- chromosome bearing sperm. This was discussed early in U.S. Pat. No. 4,362,246 and significantly expanded upon through the techniques disclosed by Lawrence Johnson in U.S. Pat. No. 5,135,759. The Johnson technique of utilizing flow cytometry to separate X- and Y- chromosome bearing sperm has been so significant an advancement that it has for the first time made the commercial separation of such sperm feasible. While still experimental, separation has been significantly enhanced through the utilization of high speed flow cytometers such as the MoFlo.RTM. flow cytometer produced by Cytomation, Inc. and discussed in a variety of other patents including U.S. Pat. Nos. 5,150,313, 5,602,039, 5,602,349, and 5,643,796 as well as international PCT patent publication WO 96/12171. While the utilization of Cytomation's MoFlo.RTM. cytometers has permitted great increases in speed, and while these speed increases are particularly relevant given the high number of sperm often used, certain problems have still remained. In spite of the almost ten-fold advances in speed possible by the MoFlo.RTM. flow cytometer, shorter and shorter sorting times have been desired for several reasons. First, it has been discovered that as a practical matter, the sperm are time-critical cells. They lose their effectiveness the longer they remain unused. Second, the collection, sorting, and insemination timings has made speed an item of high commercial importance. Thus, the time critical nature of the sperm cells and the process has made speed an essential element in achieving high efficacy and success rates.

BSPR:

In addition to the delicateness of the process, it has always been known that the sperm themselves are extremely delicate cells. While this factor at first glance seems like it might be considered easily understood, in fact, the full extent of the cells' sensitivities have not yet been fully explored. In the context of flow cytometry in general, most sorted cells or particles have often been spherical or otherwise physically able to withstand a variety of abuses. This is not the case for sperm cells. In fact, as the present invention discloses, the processing through normal flow cytometer techniques may, in fact, be unacceptable for cytometric sorting of sperm cells in certain applications. The sensitivities range from dilution problems and the flow cytometer's inherent need to isolate and distinguish each cell individually as well as the pressure and other stresses which typical flow cytometry has, prior to the present invention, imposed upon the cells or other substances

that it was sorting. This may also represent a unique factor for sperm cells because it appears that even though the sperm cell may appear to pass through the flow cytometer and be sorted with no visually discernable side-effects, in fact, the cells themselves may have been stressed to the point that they perform less than optimally in the insemination process. Thus, an interplay of factors seems involved and has raised unusual problems from the perspective of sperm cell sorting and ultimate use for artificial insemination.

BSPR:

Another problem which has been faced by those in the industry--again, in spite of the great advances by the Johnson patent and related technology--is the fact that the problem itself, namely, artificial insemination with a high success rate is one of a statistical nature in which a multitude of factors seem to interplay. Thus, the solutions proposed may to some degree involve a combination of factors which, when thoroughly statistically studied, will be shown to be necessary either in isolation or in combination with other factors. Such a determination is further compounded by the fact that the results themselves vary by species and may be difficult to ascertain due to the fact that testing and statistical sampling on a large enough data base is not likely to be worth the effort at the initial stages. For these reasons the invention can also involve a combination of factors which may, individually or in combination, represent the appropriate solutions for a given application. This disclosure is thus to be considered broad enough so that the various combinations and permeations of the techniques disclosed may be achieved. Undiscovered synergies may exist with other factors. Such factors may range from factors within the sorting or flow cytometer steps to those in the collection as well as insemination steps. At present, studies have been primarily achieved on bovine species, however, it is not believed that these techniques will be limited to such species or, for that matter to only sperm cells. It appears that the techniques used may have application beyond just sperm cells into areas which involve either sensitive items to be sorted or merely minimization of the impacts of the stresses of flow cytometry upon the item sorted.

DEPR:

As mentioned, the basic goal is that of separating the X-bearing sperm from the Y-bearing sperm. This is done in a manner which isolates the two types of sperm so that each can be separately packaged and dealt with. The isolation is preferably done through the use of flow cytometry. Flow cytometry in general is a technique which is well understood. For instance, the basic aspects of it are shown and discussed in a variety of patents to Cytomation, Inc. such as the U.S. Patents and other publications listed earlier. Each of these patents and the references cited therein, are incorporated by reference, thus those skilled in the art can easily understand the basic principles involved.

DEPR:

Essentially, flow cytometry involves sorting items, such as cells, which are provided to the flow cytometer instrument through some type of cell source. A conceptual instrument is shown in FIG. 1. The flow cytometer instrument includes a cell source (1) which acts to establish or supply cells or some other type of item to be analyzed by the flow cytometer. The cells are deposited within a nozzle (2) in a manner such that the cells are surrounded by a sheath fluid (3). The sheath fluid (3) is usually supplied by some sheath fluid source (4) so that as the cell source (1) supplies its cells, the sheath fluid (3) is concurrently fed through the nozzle (2). In this manner it can be easily understood how the sheath fluid (3) forms a sheath fluid environment for the cells. Since the various fluids are provided to the flow cytometer at some pressure, they flow out of nozzle (2) and exit at the nozzle orifice (5). By providing some type of oscillator (6) which may be very precisely controlled through an oscillator control (19), pressure waves may be established within the nozzle (2) and transmitted to the fluids exiting the nozzle (2) at nozzle orifice (5). Since the oscillator (6) thus acts upon the sheath fluid (3), the stream (7) exiting the nozzle orifice (5) eventually and regularly forms drops (8). Because the cells are surrounded by a sheath fluid environment, the drops (8) may contain within them individually isolated

(generally) cells or other items.

DEPR:

One of the aspects of flow cytometry which is particularly important to its application for sperm sorting is the high speed operation of a flow cytometer. Advances have been particularly made by the flow cytometers available through Cytomation, Inc. under the MoFlo.RTM. trademark. These flow cytometers have increased sorting speeds extraordinarily and have

DEPR:

thus made flow cytometry a technique which is likely to make feasible the commercial application of sperm sorting (among other commercial applications). They act to achieve high speed sorting, that is at a speed which is notably higher than those otherwise utilized. Specifically, Cytomation's MoFlo.RTM. flow cytometers act with oscillator frequencies of greater than about five kilohertz and more specifically can be operated in the 10 to 30 or even the 50 kilohertz ranges. Thus droplets are formed at very high frequencies and the cells contained within the sheath fluid environment can be emitted very rapidly from the nozzle (2). As a result, each of the components such as the nozzle (2) oscillator (6), and the like which make up and are part of a flow cytometer system result in a high speed cell sorter. In the application of a high speed cell sorter to the sorting of sperm cells, sorting at rates of greater than about 500 sorts per second is achieved. In fact, rates of sorting in the thousand and twelve hundred ranges have already been achieved through a high speed cell sorter. Importantly, it should be understood that the term "high speed" is a relative term such that as other advances in flow cytometry and specific applications are achieved, the aspect which is considered "high" may be varied or may remain absolute. In either definition, the general principle is that the sorting may occur at rates at which the parameters and physical characteristics of the flow cytometer are significant to the cells themselves when sorting particular cells such as sperm cells.

DEPR:

For the sheath fluid, a substance is selected according to one embodiment of the invention so that it may be chemically coordinated to prevent minimal changes. Thus, by selecting the appropriate sheath fluid not only in context of flow cytometry parameters, but rather also in context of the cell parameters themselves, the changes experienced by the cells and the over all result of the sorting can be enhanced. This is shown conceptually in FIG. 3. FIG. 3 shows some type of chemical factor (such as citrate or other factors) as it may exist throughout the various phases of the process. For instance, the four phases shown might represent the following: phase I may represent the existence of the cells within the cell source (1), phase II might show the existence of the cells as they are sorted in the sheath fluid environment, phase III might show the cells as they are collected after sorting and phase IV might show the reconstituted cells in a storage medium after sorting. These four phases as shown for the prior art may experience vastly different chemical factor environments. As shown conceptually, however, in the present invention the cells may experience very little change, most notably the dip or drop experienced between phases I and II may be virtually absent. This is as a result of the selection of the appropriate sheath fluid as mentioned above. Thus, as a result of being subjected to an appropriate sheath fluid, the cells in the present invention may experience a much lower level of stress.

DEPR:

The process of collecting, sorting, and eventually inseminating an animal through the use of flow cytometry involves a variety of steps. In the context of bovine insemination, first the semen is collected from the bull through the use of an artificial vagina. This occurs at rates of approximately 1.5 billion sperm per ml. This neat semen may be checked through the use of a spectrophotometer to assess concentration and may be microscopically evaluated to assure that it meets appropriate motility and viability standards. Antibiotics may then be added. As a result the initial sample may have approximately 60 to 70 percent of the progressively motile sperm per ejaculate. For processing, a dilution through of some type TALP (tyrode

albumin lactate pyruvate) may be used to get the numbers of sperm at a manageable level (for flow analysis) of approximately 100 million per ml. The TALP not only nurtures the sperm cells, but it may make them hyper-activated for the staining step. Prior to staining, in some species such as the equine species, centrifugation may be accomplished. Staining may be accomplished according to a multi-stained or single-stained protocol, the latter, the subject of the Johnson Patent and related technology. The staining may be accomplished while also adjusting the extender to create the appropriate nutrient environment. In bovine applications this may involve adding approximately 20% egg yolk content in a citrate solution immediately after staining. Further, in staining the sperm cells, it has been discovered that by using higher amounts of stain than might to some extent be expected better results may be achieved. This high concentration staining may involve using amounts of stain in the tens of micro-molar content such as discussed in the examples below where 38 micro-molar content of Hoechst 33342 stain was used.

DEPR:

After adding the stain, an incubation period may be used such as incubating at one hour at 34.degree. C. to hasten the dye uptake with concentrations at about 100 million sperm cells per ml. Filtration may then be accomplished to remove clumps of sperm cells and then dilution or extending may or may not be accomplished to the desired sort concentration of approximately 100 million sperm cells per ml may be accomplished. Sorting according to the various techniques discussed earlier may then be accomplished from which sperm cells may be recovered in the collection phase. As mentioned earlier, the collection may result in samples with approximately 2% egg yolk citrate concentrate content (for bovine species). This sample may then be concentrated to about 3-5 million sperm cells per ml through the use of centrifugation after which the sheath fluid and preserving fluid may be removed. A final extension may then be accomplished with either 20% egg yolk citrate or a Cornell Universal Extender or the like. The Cornell Universal Extender may have the following composition for 1000 ml:

DEPR:

By example, twelve Angus crossbred heifers were superovulated using standard procedures: 6, 6, 4, 4, 2, 2, 2, and 2 mg FSH were injected intramuscularly at half-day intervals beginning between days 9 and 12 of the estrous cycle; 25 and 12.5 mg prostaglandin F-2 alpha were injected intramuscularly with the 6th and 7th FSH injections. Sperm from bulls of unknown fertility were stained with Hoechst 33342 and then sorted using a MoFlo.RTM. flow cytometer/cell sorter yielding 700-800 live sperm of each sex/sec. Average sort purity was 89% of the desired sex. Sorted sperm were concentrated to 3.36.times.10.sup.6 sperm/ml by centrifugation at 650 g for 10 min, cooled to 5.degree. C., and stored 4 h. Then 184 ul were loaded in 0.25 ml plastic straws; half the dose was inseminated into each uterine horn 20 to 24 h post-onset of estrus using automatic side-opening embryo transfer sheaths. Embryos were collected by standard non-surgical procedures at 7 or 16 days post-estrus. Results were similar between day 7 and 16 collections and between X- and Y-sorted sperm. Embryos were recovered from 9 heifers. There were 52 embryos (mean, 4.3+-.5.3/donor) at normal stages of development, 13 retarded embryos and 31 unfertilized ova. Forty-six embryos were sexed by PCR using primers for a Y-chromosome-specific DNA sequence; 43 (93%) were of the intended sex. Although this study involved few animals, surprisingly, insemination of superovulated heifers with only 600,000 total (live) sexed unfrozen sperm gave similar results to conventional procedures. Variations on the above may also be accomplished, including, but not limited to, sorting through other than flow cytometric means, achieving superovulation in other manners, increasing fertility in other manners, and the like.

DEPR:

Angus heifers, 13-14 mo of age and in moderate body condition, were synchronized with 25 mg of prostaglandin F-2 alpha at 12-day intervals and inseminated 6-26 h after observed standing estrus. Freshly collected semen from three 14-26 mo old bulls was incubated in 38 .mu.M Hoechst 33342 at 75.times.10.sup.6 sperm/ml in a TALP medium for 1 h at 34.degree. C. Sperm

were sorted by sex chromosomes on the basis of epifluorescence from laser excitation at 351 and 364 nm at 150 mW using a MoFlo.RTM. flow cytometer/cell sorter operating at 50 psi and using 2.9% Na citrate as sheath fluid. X chromosome-bearing sperm (.about.90% purity as verified by resorting sonicated sperm aliquots) were collected at .about.500 live sperm/sec into 2-ml Eppendorf tubes containing 100 .mu.l Cornell Universal Extender (CUE) with 20% egg yolk. Collected sperm were centrifuged at 600.times.g for 10 min and resuspended to 1.63.times.10.sup.6 live sperm/ml in CUE. For a liquid semen unsexed control; Hoechst 33342-stained sperm were diluted with sheath fluid to 9.times.10.sup.5 sperm/ml and centrifuged and resuspended to 1.63.times.10.sup.6 progressively motile sperm/ml in CUE. Sexed semen and liquid control semen were cooled to 5.degree. C. over 75 min and loaded into 0.25-ml straws (184 ul/straw). Straws were transported at 3 to 5.degree. C. in a temperature-controlled beverage cooler 240 km for insemination 5 to 9 h after sorting. Sexed semen and liquid control semen were inseminated using side-opening blue sheaths (IMV), one half of each straw into each uterine horn (3.times.10.sup.5 live sperm/heifer). As a standard control, semen from the same bulls had been frozen in 0.5-cc straws by standard procedures (mean 15.6.times.10.sup.6 motile sperm/dose post-thaw), thawed at 35.degree. C. for 30 sec, and inseminated into the uterine body. Treatments were balanced over the 3 bulls and 2 inseminators in a ratio of 3:2:2 inseminations for the sexed semen and two controls. Pregnancy was determined ultrasonically 31-34 days after insemination and confirmed 64-67 days later when fetuses also were sexed (blindly). Data are presented in the table.

DEPR:

The objective was to determine pregnancy rates when heifers are inseminated with extremely low numbers of frozen sperm under ideal field conditions. Semen from three Holstein bulls of above average fertility was extended in homogenized milk, 7% glycerol (CSS) extender plus 5% homologous seminal plasma to 2.times.10.sup.5, 5.times.10.sup.5 or 10.times.10.sup.6 (control) total sperm per 0.25 ml French straw and frozen in moving liquid nitrogen vapor. Semen was thawed in 37.degree. C. water for 20 sec. Holstein heifers 13-15 mo of age weighing 350-450 kg were injected with 25 mg prostaglandin F-2-alpha (Lutalyse.RTM.) twice at a 12-day interval and inseminated with an embryo transfer straw gun and side-opening sheath, half of the semen deep into each uterine horn 12 or 24 h after detection of estrus. The experiment was done in five replicates over 5 months, and balanced over two insemination technicians. Ambient temperature at breeding was frequently -10 to -20.degree. C., so care was taken to keep insemination equipment warm. Pregnancy was determined by detection of a viable fetus using ultrasound 40-44 days post-estrus and confirmed 55-62 days post-estrus; 4 of 202 conceptuses were lost between these times. Day 55-62 pregnancy rates were 55/103 (53%), 71/101, (70%), and 72/102 (71%) for 2.times.10.sup.5, 5.times.10.sup.5 and 10.times.10.sup.6 total sperm/inseminate ($P<0.1$). Pregnancy rates were different ($P<0.05$) among bulls (59, 62, and 74%), but not between technicians (64 and 65%) or inseminations times post-estrus (65% for 12 h and 64% for 24 h, $N=153$ at each time). With the methods described, pregnancy rates in heifers were similar with 5.times.10.sup.5 and 10.times.10.sup.6 total sperm per inseminate.

DEPR:

Semen was collected from bulls at Atlantic Breeders Cooperative, diluted 1:4 with a HEPES-buffered extender +0.1% BSA, and transported 160 km (.about.2 HR) to Beltsville, Md. where it was sorted at ambient temperature by flow cytometry into a TEST yield (20%) extender using methods described previously (Biol Reprod 41:199). Sorting rates of up to 2.times.10.sup.6 sperm of each sex per 5-6 h at .about.90% purity were achieved. Sperm were concentrated by centrifugation (300 g for 4 min) to 2.times.10.sup.6 sperm/ml. Some sperm were sorted into extender containing homologous seminal plasma (final concentration, 5%). Sorted sperm were shipped by air to Colorado (.about.2,600 km) and stored at either ambient temperature or 5.degree. C. (cooled during shipping over 6 hr in an Equitainer, an insulated device with an ice-containing compartment). Heifers or dry cows detected in estrus 11 to 36 h earlier were inseminated within 9 to 29 h of the end of the sperm sorting session. Sperm (1 to 2.times.10.sup.5 in 0.1 ml) were deposited deep in the

uterine horn ipsilateral to the ovary with the largest follicle as determined by ultrasound at the time of insemination.

DEPR:

To summarize, it is possible to achieve pregnancies in cattle via artificial insemination of sperm sorted for sex chromosomes by flow cytometry, and the sex ratio of fetuses approximates that predicted by reanalysis of sorted sperm for DNA content (90%). However, pregnancy rates varied greatly in these preliminary experiments which required shipping sperm long distances. Fertility decreased drastically by 17 h post-sorting, but there was some confounding because different bulls were used at the different times. Further studies are needed to determine whether variation observed in pregnancy rates was due to bull differences, insemination techniques, interval between sorting and insemination, or other factors.

DETL:

I. FINAL COMPOSITION: 80% sodium citrate solution (72 mM) 20% (vol/vol) egg-yolk II. PREPARATION FOR 1 LITER:
A. Sodium citrate solution 1. Place 29.0 grams of sodium citrate dihydrate (Na.sub.3 C.sub.6 H.sub.5 O.sub.7.2H.sub.2 O) in a 1,000 ml volumetric flask 2. Add deionized or Nanopure water to make 1,000 ml final volume. 3. Transfer to bottles and autoclave at 15 lbs pressure (245.degree. F.) for at least 30 minutes. a. Autoclave solution using conditions to minimize evaporation (loose cover) b. Be careful that water does not boil away. 4. Cool slowly at room temperature. 5. Store sealed in a 5.degree. C. cold room. B. Egg preparation 1. Obtain fresh hen's eggs from a good commercial source. 2. Wash the eggs free of dirt (do not use too much detergent) and rinse. 3. Immerse eggs in 70% ethanol for 2-5 minutes. 4. Remove eggs and allow to dry (or wipe dry) and store on a clean towel. C. Preparation of extender 1. Use sterile, clean glassware 2. A-fraction (non-glycerol fraction) a. Place 800 ml of 2.9% sodium citrate solution in a 1,000 ml graduated cylinder. b. Antibiotic levels for the non-glycerol containing fraction (A-fraction) of the extender may be as follows: I. Tylosin = 100 .mu.g/ml ii. Gentamicin = 500 .mu.g/ml iii. Linco-spectin = 300/600 .mu.g/ml c. Add 200 ml of fresh egg-yolk as outlined below (Section D) I. Mix very thoroughly. d. This provides A-fraction extender based on 2.9% sodium citrate, with 20% egg-yolk and antibiotics at concentrations known to be non-toxic to bull sperm. e. Extender can be stored overnight at 5.degree. C. f. Decant supernatant (upper 800 ml) the next day. g. Warm to 37.degree. C. prior to use the next day. D. To add egg-yolk to a buffered solution, the following procedure works well. 1. Wash egg and clean the eggs (see B above) 2. Open egg and separate yolk from albumin using a yolk separator. Alternatively, pour yolk back and forth 2-3 times between the two half shells. Do not rupture the membrane around the yolk. 3. Place the yolk onto a sterile piece of 15 cm filter paper. 4. Hold the filter paper over the graduated cylinder containing buffer and squeeze the yolk (rupturing the membrane) and allow the yolk to run out of the golded filter paper into the cylinder. Typically about 12-15 ml of the yolk can be obtained from one egg.

CCOR:

435/2

ORPL:

Rath, D., et al., "Production of Piglets Preselected for Sex Following in Vitro Fertilization with X and Y Chromosome-Bearing Spermatozoa Sorted by Flow Cytometry", Theriogenology, 47, 1997, pp 795-800.

ORPL:

Squires, E., "Simultaneous Analysis of Multiple Sperm Attributes by Flow Cytometry", Diagnostic Techniques and Assisted Reproductive Technology, The Veterinary Clinics of North America, Equine Practice, vol. 12, No. 1, Apr. 1996, pp 127-130.

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L6: Entry 11 of 20

File: USPT

Nov 25, 1997

DOCUMENT-IDENTIFIER: US 5691133 A

TITLE: Method to quickly add cryoprotectants to sperm cells while maintaining viability

BSPV:

(1) the method is usable for other cryoprotectants besides the commonly used glycerol,

DEPR:

These equations respectively describe total membrane volume flux ($J_{sub.v}$) and transmembrane permeable solute flux ($J_{sub.CPA}$). Assuming the solution consists of a single permeable solute (here, CPA) and other impermeable solutes, the mathematical equations are: ##EQU1## where $J_{sub.v}$ =total volume flux, V =sperm volume, t =time, N =mole number of solute, $A_{sub.c}$ =sperm surface area, $L_{sub.p}$ =water permeability coefficient of the sperm membrane, C =concentration of solute, $J_{sub.CPA}$ =CPA flux across the cell membrane, superscript e =extracellular, superscript i =intracellular, $C_{sub.CPA}$ =average CPA concentration of extracellular and intracellular concentrations. Furthermore, R =gas constant, T =absolute temperature, $P_{sub.CPA}$ =CPA permeability coefficient of the sperm membrane, and σ =the reflection coefficient of the particular CPA. The reflection coefficient is generally specific to a particular cryoprotectant and represents the opposing actions of water and cryoprotectant moving in opposite directions through the sperm cell membrane. Typically it is assumed to be 1. For example, using glycerol as the CPA, a value of 0.7 to 1 has only insignificant effect on the predictions found using this model. However, it must be remembered that its exact value is dependent on the cryoprotectant utilized and is a point where the user may fine tune the model for their particular use.

DEPR:

Examples of the physical values for human sperm needed are shown in Table 1. Similar values for other species of sperm are within the skill in the art to obtain. For example, the permeability coefficient of the cryoprotective agent can be determined using procedures as disclosed in Glycerol Permeability of Human Spermatozoa and its Activation Energy, by Gao, Mazur, Kleinhans, Watson, Noiles, and Critser, Cryobiology 29, 657-667, the disclosure of which is hereby incorporated by reference into this specification; or, the permeability coefficient of water can be determined using procedures as disclosed in Determination of Water Permeability Coefficient for Human Spermatozoa and its Activation Energy, by Noiles, Mazur, Watson, Kleinhans, and Critser; Biology of Reproduction 48, 99-109 (1993) the disclosure of which is also hereby incorporated by reference into this specification.

DEPR:

The human semen used in this example was obtained by masterbation from healthy donors after at least two days of sexual abstinence. The samples were allowed to liquefy in an incubator for 1 hour, at 37.degree. C., in high humidity, and in 5% $CO_{sub.2}$ and 95% air. A swim-up procedure was performed to separate motile sperm from immotile sperm. The motile sperm suspensions were centrifuged at 400.times.g for 7 minutes and then were resuspended in isotonic TL-Hepes medium, that is, HEPES-buffered TALP medium (286-290 mOsm) supplemented with Pyruvate (0.01 mg/ml) and BSA (4 mg/ml), at a cell concentration of 1.times.10.sup.9 sperm/ml.

DEPR:

A similar pattern, with the sperm's volume first shrinking or swelling and then recovering, takes place during the CPA addition or removal process from the sperm. Generally, when a cell is placed in a solution that is hyperosmotic with respect to the permeating solute (e.g. glycerol) but isotonic with respect to the impermeable salts, it first shrinks because of the osmotic efflux of intracellular water and then increases in volume as the solute (e.g. glycerol) permeates and as water concomitantly reenters the cell. When cells with CPA are exposed to an isotonic solution, they will swell because of osmotic influx of extracellular water and then decrease in volume as the CPA diffuses out of the sperm and as water concomitantly moves out.

DEPR:

The sperm's volume changes during a 1-step addition and a 1-step removal of 9.5-2M glycerol were calculated from computer simulation and are respectively shown in FIGS. 7 and 8. The higher the glycerol concentration, the longer the time period taken for the sperm volume recovery. However, typically it takes but a few seconds for human sperm to achieve their osmotic equilibration volumes. This means that the sperm experience the shrunken or swollen states for only an order of seconds during the addition or removal of glycerol. Therefore, the information concerning post-anisomotic tolerance of the sperm returned to isotonic conditions after a short time exposure (an order of seconds) to the anisomotic conditions is particularly important for designing optimal CPA addition/removal procedures to prevent sperm osmotic injury.

DEPR:

The kinetics of water and glycerol transport across the sperm membrane were modeled using standard computing techniques that are well within the skill of the art of this invention and using Equations 1 to 4. Two preferred schemes of CPA addition, Fixed-Volume-Step (FVS) and Fixed-Molarity-Step (FMS), were analyzed in this regard and are presented below.

DEPR:

Referring to FIG. 10, there is shown the calculated sperm volume change arising during a one-step, a two-step, and a four-step addition of glycerol to achieve a final 1M glycerol concentration at 22.degree. C. using an FMS addition.

DEPR:

Referring to FIG. 11, there is shown a comparison between a four-step FVS addition of glycerol and a four-step FMS of glycerol.

DEPR:

From FIGS. 9 to 11, a one-step addition of glycerol to sperm is predicted to cause a 10% to 20% sperm motility loss. This loss is predicted to occur because the minimum volume which the sperm can attain during this addition is approximately 72% of the original cell volume, a value less than the minimum acceptable LVL of 75% previously determined above. In contrast, a four-step FVS or FMS addition of glycerol was predicted to greatly reduce such sperm motility loss (less than 5%).

DEPR:

Referring to FIG. 12, there is shown a one-step removal of glycerol. This removal was predicted to cause as high as 70% motility loss because the maximum cell volume during the glycerol removal was calculated to be over 1.6 times larger than the isotonic cell volume, much higher than the upper volume limit of the sperm.

DEPR:

Referring to FIG. 14, there is shown a comparison between the eight-step FMS and the eight-step FVS removal procedures. The eight-step FMS removal was predicted to prevent sperm motility loss over the FVS procedure. An eight-step FVS removal was predicted to cause a maximum cell swelling of over 1.2 times higher than isotonic cell volume while FMS removal was predicted to be much

lower than the UVL, indicating that the eight-step FVS removal is not as good as eight-step FMS removal. Also apparent from the computer simulation was the prediction that the human sperm would rapidly achieve an osmotic equilibrium (within seconds) during the 1-step or stepwise glycerol addition or removal. This further indicated that only a short time interval between steps of glycerol addition or removal was required.

DEPR:

In sum, a four-step FMS addition and an eight-step FMS removal of glycerol were predicted to be optimal protocols to prevent sperm motility loss in human sperm using the foregoing equations.

DEPR:

A two-step removal of CPA from the cells using a nonpermeating solute as an osmotic buffer has been previously used to avoid the osmotic injury to other cell-types. The detailed procedure is: (1) the CPA is directly removed from the cells by transferring cells to a hyperosmotic medium (osmotic buffer) containing no CPA but only nonpermeating solutes, and then (2) the cells are directly transferred to an isotonic solution. It has been known that 600 mOsm is the hyperosmotic upper tolerance limit for human sperm (FIG. 9). Therefore, osmolality of the osmotic buffer medium should not be over 600 mOsm. Under this limit, a hyperosmolality of 600 mOsm is expected to provide the maximum "buffer effect" to reduce the sperm volume swelling during the first step of the "two-step" procedure for glycerol removal. Sperm volume change during the 2-step glycerol (1M) removal process using a 600 mOsm buffer medium was calculated and shown in FIG. 15. It was predicted that maximum volume the sperm would achieve 1.25 times of the isotonic cell volume, which is higher than the UVL of the sperm, and might cause over 50% sperm motility loss.

DEPR:

TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:

Add 2000 .mu.l of isotonic solution directly to 100 .mu.l of cell suspension with 1M glycerol.

DEPR:

Glycerol was experimentally added to or removed from the human sperm using the stepwise procedures as predicted from the computer simulation. Percent motility of the human sperm after one step or a four-step FMS addition of glycerol (Table 2) is shown in FIG. 16. One step addition created approximately 20% sperm motility loss while the four-step addition, less than 8%. FIG. 17 shows effects of different glycerol removal procedures (Table 3) on the human sperm motility loss. Less than 30% motile sperm kept motility after one-step removal of glycerol while majority of sperm (over 92%) maintained the motility after the eight-step FMS removal. The motility loss caused by a two-step removal of glycerol using sucrose as a non-permeating buffer (total osmolality of the buffer medium was 600 mOsm) was close to 45%. Thirty-five percent of the sperm lost motility after a eight-step FVS removal of glycerol. The experimental results agree well with the predictions generated from computer simulation.

DEPR:

FIG. 18 shows the membrane integrity of the human sperm in 1M glycerol solution or after addition and removal of 1M glycerol by the different procedures. The sperm appeared to maintain membrane integrity under all

experimental conditions.

DEPR:

This example presented two CPA addition or removal schemes (FVS and FMS). As the example shows, the Fixed-Molarity-Step is preferable to reduce osmotic injury over the Fixed-Volume-Step. In particular, for human sperm, the example shows that a four-step FMS addition of glycerol to the sperm and an eight-step FMS removal of glycerol from the sperm were predicted to be optimal, which was confirmed upon implementation. Upon reviewing each scheme, the minimum/maximum cell volumes after each step of FVS addition/removal was uneven or unequal, some of which exceeded the LVL and UVL of the sperm. In contrast, minimum/maximum cell volumes after each step of FMS addition/removal of glycerol were shown to be relative even (FIGS. 11 and 14). For a fixed number of steps, the extent of cell volume change during CPA addition/removal using the FMS scheme is much smaller than that using the FVS scheme (also see FIGS. 11 and 14).

DEPL:

where $M_{sub.s}$ = CPA concentration in sperm suspension (molarity) after $n-1$ step dilutions, $M_{sub.o}$ = CPA concentration in initial sperm suspension (molarity), n = total number of steps, i = the i th step addition, $V_{sub.o}$ = original volume of sperm suspension (ml) and $V_{sub.i}$ = volume of isotonic solution added into the sperm suspension at the i th step. After $n-1$ steps of adding isotonic solution into the sperm suspension, the diluted sperm suspension is centrifuged, for example, at 400 g for 5-7 minutes., and then the sperm pellet is resuspended in isotonic solution to make the last (n th) step dilution.

DEPL:

Glycerol permeability coefficient (P_s) 1.1×10^{-3} cm/minutes (22.degree. C.)

DEPL:

Table 4. Procedures used in 2-step removal of 1M glycerol from the human sperm using sucrose as an osmotic buffer

DEPC:

Prediction of Optimal Conditions for Glycerol Addition or Removal

DEPV:

(1) Add 2000 .mu.l of sucrose buffer medium (TALP+sucrose, 600 mOsm) to 100 .mu.l of sperm suspension with 1M glycerol; (2) centrifuge the suspension (400 g for 7 minutes) and take off the supernatant; and (3) resuspend cell pellet with 500 ul isotonic TALP medium.

DETL:

TABLE 2 _____ Procedures used in 4-step addition of 1 ml of 2 M glycerol solution of 1 ml of isotonic sperm suspension
FVS FMS _____ Add 0.25 ml of 2 M glycerol
Stepwise add 0.14, 0.19, 0.27 4 times to 1 ml isotonic and 0.4 ml of 2 M glycerol sperm suspension to isotonic sperm suspension

DETL:

TABLE 3 _____ Procedures used in 1-step and 8-step removal of 1 M glycerol from the human sperm 8-Step Dilution FVS FMS
_____ Add 100 .mu.l of isotonic TALP Stepwise
add 14.3, 19, 26.6, 7 times to sperm suspension to and 40 .mu.l of isotonic TALP achieve a final glycerol medium to 100 .mu.l of sperm sus- concentration, 0.125 M. pension with 1 M glycerol; (2) After centrifugation, 710 centrifuge the cell suspension .mu.l of supernatant was taken at 400 g for 5-7 minutes.; (3) off. Remaining cell sus- take off 170 .mu.l of the super- pension volume is 90 .mu.l natant; Stepwise add 10, 20 and 60 .mu.l of isotonic solution to the remaining 30 ul of sperm suspension. After the above 7 steps dilution, the glycerol concentration in the sperm suspension is 0.125 M. The final suspension volume is 90 .mu.l _____

CLPR:

4. The method of claim 1, wherein said cryoprotectant comprises glycerol.

CLPR:

12. The method of claim 11, wherein said cryoprotectant is selected from the group consisting of dimethyl sulfoxide, glycerol, and ethylene glycol.

CCOR:

435/2

ORPL:

Cryobiology, vol. 26, issued 1989, Fiser et al., "The Effect of Glycerol-Related Osmotic Changes on Post-Thaw Motility and Acrosomal Integrity of Ram Spermatozoa", pp. 64-69.

ORPL:

Cryobiology, vol. 29, issued 1992, Gao et al., "Glycerol Permeability of Human Spermatozoa and its Activation Energy", pp. 657-667.

ORPL:

Biology of Reproduction, vol. 49, issued 1993, Gao et al., "Hyperosmotic Tolerance of Human Spermatozoa: Separate Effects on Glycerol, Sodium Chloride, and Sucrose on Spermolysis", pp. 112-113.

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Dec 23, 1997

File: USPT

L6: Entry 10 of 20

DOCUMENT-IDENTIFIER: US 5700632 A
TITLE: General method to quickly remove cryoprotectants from animal cells while maintaining viability

BSPU:
(1) the method is usable for other cryoprotectants besides the commonly used glycerol, dimethyl sulfoxide, or ethylene glycol,

DEPR:
The first two equations respectively describe total membrane volume flux (J.sub.v) and transmembrane permeable solute flux (J.sub.CPA). Assuming the solution consists of a single permeable solute (here, CPA) and other impermeable solutes (i.e. salt), the mathematical equations are: ##EQU1## where J.sub.v =total volume flux, V=cell volume, t=time, N=mole number of the solute, A.sub.c =cell surface area, L.sub.p =water permeability coefficient of the cell membrane, C=concentration of solute, J.sub.CPA =CPA flux across the cell membrane, superscript e=extracellular, superscript i=intracellular C.sub.CPA =average CPA concentration of extracellular and intracellular concentrations. Furthermore, R=gas constant, T=absolute temperature, P.sub.CPA =CPA permeability coefficient of the cell membrane, and .sigma.=the reflection coefficient of the particular CPA. The reflection coefficient is generally specific to a particular cryoprotectant and represents the opposing actions of water and cryoprotectant moving in opposite directions through the cell membrane. Typically it is assumed to be 1. For example, using glycerol as the CPA, a value of 0.7 to 1 has only insignificant effect on the predictions found using this model. However, it must be remembered that its exact value is dependent on the cryoprotectant utilized and is a point where the user may fine tune the model for their particular use. Using the devices and methods discussed further in this patent, a more exact value can be determined.

DEPR:
Examples of the physical values for human sperm needed are shown in Table 1. Similar values for other species of sperm are within the skill in the art to obtain. For example, the permeability coefficient of the cryoprotective agent can be determined using procedures as disclosed in Glycerol Permeability of Human Spermatozoa and its Activation Energy, by Gao, Mazur, Kleinhans, Watson, Noiles, and Critser, Cryobiology 29, 657-667, the disclosure of which is hereby incorporated by reference into this specification; or, the permeability coefficient of water can be determined using procedures as disclosed in Determination of Water Permeability Coefficient for Human Spermatozoa and its Activation Energy, by Noiles, Mazur, Watson, Kleinhans, and Critser; Biology of Reproduction 48, 99-109 (1993) the disclosure of which is also hereby incorporated by reference into this specification.

DEPR:
Human RBC Preparation: Human blood was obtained by venipuncture from three healthy donors into 10 cc vacutainer tubes with acid citrate dextrose (SCD) anticoagulant. Cells were washed three times by centrifugation at 1000.times.g for five minutes each in PBS (pH=7.4) prior to use.

DEPR:
EPR sample preparation: Two .mu.l of 50 mM tempone, 10 m .mu.l of 250 mM CrOx, 28 .mu.l of sodium chloride solution (83, 153, 345, 694, 1217, or 2270 mOsm),

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and 10 μ l packed sperm cells in TALP (centrifuged at 400.times.g for 10 minutes) were combined in a 5 ml sterile culture tube. The combined solution had a total volume of 50 μ l containing final concentrations of 2 mM tempone, 50 mM CrOx, and a total osmotic concentration of 250, 290, 400, 600, 900, or 1500 mOsm. The contents were mixed manually by tapping the tube. Ten μ l of each sample was transferred to another tube for trypan blue staining (10 μ l sample and 10 μ l 2% trypan blue in isotonic saline, incubated for 10 min.) and cell counting of membrane intact cells on a standard hemocytometer under a light microscope which yields the sample cell concentration C.sub.c. The remaining sample was drawn by capillary action into a 50 μ l disposable micropipet (Clay Adams #4622, Parsnippany, N.J.) and sealed with Crioseal (Monoject Scientific, St. Louis, Mo.) for the EPR measurement. All the final osmotic concentrations were determined by linear addition of the individual osmolalities of the sodium chloride and CrOx (non-permeating solute). The osmolality of tempone was not included in the final concentration because it is a cell membrane permeable solute and has a very low concentration. The final strength of each solution was checked on a freezing point depression osmometer (Advanced DigiMatic Osmometer, Model 3D2) yielding agreement within 3%. The high concentration of tempone, 2 mM, was chosen to optimize signal strength and leads to some spin-spin line broadening.

DEPR:

The human semen used in this example was obtained by masturbation from healthy donors after at least two days of sexual abstinence. The samples were allowed to liquefy in an incubator for 1 hour, at 37.degree. C., in high humidity, and in 5% CO.sub.2 and 95% air. A swim-up procedure was performed to separate motile sperm from immotile sperm. The motile sperm suspensions were centrifuged at 400.times.g for 7 minutes and then were resuspended in isotonic TL-Hepes medium, that is, HEPES-buffered TALP medium (286-290 mOsm) supplemented with Pyruvate (0.01 mg/ml) and BSA (4 mg/ml), at a cell concentration of 1.times.10.sup.9 sperm/ml.

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A similar pattern, with the sperm's volume first shrinking or swelling and then recovering, takes place during the CPA addition or removal process from the sperm. Generally, when a cell is placed in a solution that is hyperosmotic with respect to the permeating solute (e.g. glycerol) but isotonic with respect to the impermeable salts, it first shrinks because of the osmotic efflux of intracellular water and then increases in volume as the solute (e.g. glycerol) permeates and as water concomitantly reenters the cell. When cells with CPA are exposed to an isotonic solution, they will swell because of osmotic influx of extracellular water and then decrease in volume as the CPA diffuses out of the sperm and as water concomitantly moves out.

DEPR:

The sperm's volume changes during a 1-step addition and a 1-step removal of 9.5-2M glycerol were calculated from computer simulation and are respectively shown in FIGS. 7 and 8. The higher the glycerol concentration, the longer the time period taken for the sperm volume recovery. However, typically it takes but a few seconds for human sperm to achieve their osmotic equilibration volumes. This means that the sperm experience the shrunken or swollen states for only an order of seconds during the addition or removal of glycerol. Therefore, the information concerning post-anisomotic tolerance of the sperm returned to isotonic conditions after a short time exposure (an order of seconds) to the anisomotic conditions is particularly important for designing optimal CPA addition/removal procedures to prevent sperm osmotic injury.

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The kinetics of water and glycerol transport across the sperm membrane were modeled using standard computing techniques that are well within the skill of the art of this invention and using Equations 1 to 4. Two preferred schemes of CPA addition, Fixed-Volume-Step (FVS) and Fixed-Molarity-Step (FMS), were analyzed in this regard and are presented below.

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Referring to FIG. 11, there is shown a comparison between a four-step FVS addition of glycerol and a four-step FMS of glycerol.

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From FIGS. 9 to 11, a one-step addition of glycerol to sperm is predicted to cause a 10% to 20% sperm motility loss. This loss is predicted to occur because the minimum volume which the sperm can attain during this addition is approximately 72% of the original cell volume, a value less than the minimally acceptable LVL of 75% previously determined above. In contrast, a four-step FVS or FMS addition of glycerol was predicted to greatly reduce such sperm motility loss (less than 5%).

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either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:

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This example presented two CPA addition or removal schemes (FVS and FMS). As the example shows, the Fixed-Molarity-Step is preferable to reduce osmotic injury over the Fixed-Volume-Step. In particular, for human sperm, the example shows that a four-step FMS addition of glycerol to the sperm and an eight-step FMS removal of glycerol from the sperm were predicted to be optimal, which was confirmed upon implementation. Upon reviewing each scheme, the minimum/maximum cell volumes after each step of FVS addition/removal was uneven or unequal, some of which exceeded the LVL and UVL of the sperm. In contrast, minimum/maximum cell volumes after each step of FMS addition/removal of glycerol were shown to be relative even (FIGS. 11 and 14). For a fixed number of steps, the extent of cell volume change during CPA addition/removal using the FMS scheme is much smaller than that using the FVS scheme (also see FIGS. 11 and 14).

DEPR:

A corresponding computer program using a finite difference method to calculate the above equations is used to simulate the mass transfer process in the system. The values of f , L_2 , $C_{sub.f}$, $C_{sub.i}$, L_1 , D_1 , D_2 and $h_{sub.D}$ can be used as control factors or adjustable parameters in the computer simulation. Using this computer program coupled with the computer program to calculate cell membrane transport (described by cell membrane transport equations 1 to 4, presented earlier), one may calculate the kinetic decrease of CPA concentration in the cell suspension and in cells as well as corresponding cell volume expansion. The optimal parameters to be used to design the system are simply those combinations which allow the quickest removal of the CPA but do not cause cell volume excursion beyond cell tolerance limits. One set of acceptable values of parameters for designing the diffusion washing device for human sperm was predicted from computer simulations: pore size: 0.65 . μ m in diameter, 70% pore area, 0.5 mm of the chamber space, i.e. L value. Calculated kinetic changes of glycerol concentration (initial 1M) at the chamber position

A (center, see FIG. 19) and B (internal surface of porous membrane, see FIG. 19) are shown in FIG. 21, and the corresponding cell volume changes are calculated and predicted to be below the cell tolerance limit (1.1 times isotonic sperm volume), as shown in FIG. 22. Using a diffusion device design based on the computer simulation results above, over 95% of 1M glycerol was removed from the human sperm cells within 10 minutes without significant cell clumping, loss in motility or membrane integrity, or curling of sperm tails; this result was consistent with theoretical predictions.

DEPL:

where $M_{sub.s}$ = CPA concentration in cell suspension (molarity) after n-1 step dilutions, $M_{sub.o}$ = CPA concentration in initial cell suspension (molarity), n = total number of steps, i = the ith step addition, $V_{sub.o}$ = original volume of cell suspension (ml) and $V_{sub.i}$ = volume of isotonic solution added into the cell suspension at the ith step. After n-1 steps of adding isotonic solution into the cell suspension, the diluted cell suspension is centrifuged, for example, at 400 g for 5-7 minutes., and then the cell pellet is resuspended in isotonic solution to make the last (nth) step dilution.

DEPC:

Prediction of Optimal Conditions for Glycerol Addition or Removal

DEPV:

Procedures used in 2-step removal of 1M glycerol from the human sperm using sucrose as an osmotic buffer

DEPV:

(1) Add 2000 . μ .l of sucrose buffer medium (TALP+sucrose, 600 mOsm) to 100 . μ .l of sperm suspension with 1M glycerol; (2) centrifuge the suspension (400 g for 7 minutes) and take off the supernatant; and (3) resuspend cell pellet with 500 . μ .l isotonic TALP medium.

DETL:

TABLE 1 _____ Known characteristics of human spermatozoa _____ Surface area (A) 120 μ m.^{sup.2} Volume (V) 34 μ m.^{sup.3} Osmotically inactive volume ($V_{sub.b}$) 16.6 μ m.^{sup.3} Water permeability coefficient (Lp) 2.16 μ m/minutes/atm (22.degree. C.) Glycerol permeability coefficient ($P_{sub.CPA}$) 1.1 .times. 10.^{sup.}-3 cm/minutes (22.degree. C.) _____

DETL:

TABLE 2 _____ Procedures used in 4-step addition of 1 ml of 2M glycerol solution of 1 ml of isotonic sperm suspension FVS FMS _____ Add 0.25 ml of 2M glycerol Stepwise add 0.14, 0.19, 0.27 4 times to 1 ml isotonic and 0.4 ml of 2M glycerol sperm suspension to isotonic sperm suspension _____

DETL:

TABLE 3 _____ Procedures used in 1-step and 8-step removal of 1M glycerol from the human sperm 8-Step Dilution FVS FMS _____ Add 100 . μ .l of isotonic TALP Stepwise add 14.3, 19, 26.6, 7 times to sperm suspension to and 40 . μ .l of isotonic TALP achieve a final glycerol medium to 100 . μ .l of sperm concentration, 0.125M. suspension with 1M glycerol; After centrifugation, 710 (2) centrifuge the cell . μ .l of supernatant was taken suspension at 400 g for off. Remaining cell sus- 5-7 minutes.; (3) take off pension volume is 90 . μ .l 170 . μ .l of the supernatant; Stepwise add 10, 20 and 60 . μ .l of isotonic solution to the remaining 30 μ l of sperm suspension. After the above 7 steps dilution, the glycerol concentration in the sperm suspension is 0.125M. The final suspension volume is 90 . μ .l _____

CLPR:

4. The method of claim 1, wherein said cryoprotectant comprises glycerol.

CLPR:

14. The method of claim 11, wherein said cryoprotectant is selected from the group consisting of dimethyl sulfoxide, glycerol, and ethylene glycol.

CLPR:

23. The method of claim 19, wherein said cryoprotectant is selected from the group consisting of dimethyl sulfoxide, glycerol, and ethylene glycol.

CLPR:

28. The method of claim 24, wherein said cryoprotectant is selected from the group consisting of dimethyl sulfoxide, glycerol, and ethylene glycol.

CCOR:

435/2

ORPL:

Cryobiology, vol. 26, issued 1989, Fiser et al., "The Effect of Glycerol-Related Osmotic Changes on Post-Thaw Motility and Acrosomal Integrity of Ram Spermatozoa", pp. 64-69.

ORPL:

Cryobiology, vol. 29, issued 1992, Gao et al., "Glycerol Permeability of Human Spermatozoa and its Activation Energy", pp. 657-667.

ORPL:

Biology of Reproduction, vol. 49, issued 1993, Gao et al., "Hyperosmotic Tolerance of Human Spermatozoa: Separate Effects on Glycerol, Sodium Chloride, and Sucrose on Spermolysis", pp. 112-123.

WEST**End of Result Set**

Generate Collection

L12: Entry 1 of 1

File: USPT

Oct 31, 2000

DOCUMENT-IDENTIFIER: US 6140121 A

TITLE: Methods and compositions to improve germ cell and embryo survival and function

DEPR:

Extending sperm is used to resuspend a sperm pellet following isolation or washing, to dilute a semen sample, to dilute a culture of sperm, and the like. In this way, sperm are placed into a medium suitable for a variety of procedures, including culture, insemination, assays of fertilization potential as described herein, in vitro fertilization, freezing, intrauterine insemination, cervical cap insemination, and the like. The sperm may be added to the medium or the medium may be added to the sperm. Preferably, the medium contains gum guar, gum arabic, pectin or galacturonic acid, although another PCAGH may be used. In other aspects of this invention, methods are provided for the culture of such extended sperm to increase their survival during holding or culture at a range of temperatures from about room temperature (e.g., 20.degree. C.) to about body temperature (e.g., 37.degree. C. or 39.degree. C.). This includes culture of sperm in toxicity screen tests and the holding of sperm for sorting into X and Y chromosome-containing fractions by flow cytometry for generating sexed offspring. In other aspects of this invention, sperm extending medium is used for preparing sperm for direct insemination, cryopreservation, and for intracytoplasmic sperm injection (ICSI) which requires a more viscous media to slow motile sperm down for pick-up by the transfer pipette for injection into the egg. In ICSI, the medium contains PCAGH at higher levels than a routine extender medium (ie., 1% arabic acid or 5% gum arabic) to increase viscosity. A viscous solution of PCAGH also has a positive effect on sperm function by limiting membrane damage and possible chromatin breakdown during in vitro handling. Additional embodiments include encapsulation of the sperm (Munkittrick et al., J. Dairy Sci. 75:725-731) in an alginate or protamine sulfate microcapsule containing PCAGH, such as pectin at 0.05%. Encapsulation allows for shedding of sperm over an increased time frame so that insemination does not have to be as well timed with ovulation. PCAGH stabilizes sperm membranes from breakdown observed with current procedures.

WEST

Generate Collection

L13: Entry 1 of 20

File: USPT

Oct 31, 2000

DOCUMENT-IDENTIFIER: US 6140121 A

TITLE: Methods and compositions to improve germ cell and embryo survival and function

BSPR:

One common procedure used in sperm collection is washing sperm cells. Washing sperm prior to its use in assisted reproduction technologies is important for a variety of reasons. An ejaculate contains seminal plasma in addition to sperm cells, and the sugars and proteins in seminal plasma can be toxic to sperm cells after ejaculation. Also, sperm samples that have been frozen contain cryopreservation media which needs to be washed from the sperm cells prior to insemination in the female of some species, particularly birds and women. For all species, cryopreservative media cause lipid membrane peroxidation (LPO) and degeneration of the sperm after thawing. Generally, washing involves centrifuging a sample of semen or thawed sperm through a diluting wash media, which allows collection of a sperm-rich pellet. Although a very common procedure, centrifugation itself can cause sperm lipid peroxidation and membrane breakdown.

BSPR:

Once sperm have been washed or isolated, they are then extended (or diluted) in culture or holding media for a variety of uses. Existing sperm culture techniques result in losses of motile sperm and also damage sperm DNA over time in culture. Although sperm survive for days in the females of most species, sperm survival in culture is typically only half as long as that seen in vivo, and sperm from males with poor quality ejaculates may survive for even shorter time periods in culture. Much of this damage is due to lipid peroxidation of the membrane and DNA or chromatin breakdown. Sperm are extended in media for use in sperm analysis and diagnostic tests; assisted reproduction technologies, such as IVF, gamete intrafallopian transfer, or ICSI; insemination into the female; and holding prior to cryopreservation. Each of these uses for extended or diluted sperm requires a somewhat different formulation of basal medium; however, in all cases sperm survival is suboptimal outside of the female reproductive tract.

DRPR:

FIGS. 11A-11D are flow cytometry profiles of DNA from sperm frozen with PCAGH (A) and (B) or egg yolk buffer (C) and (D) extenders.

DEPR:

Extending sperm is used to resuspend a sperm pellet following isolation or washing, to dilute a semen sample, to dilute a culture of sperm, and the like. In this way, sperm are placed into a medium suitable for a variety of procedures, including culture, insemination, assays of fertilization potential as described herein, in vitro fertilization, freezing, intrauterine insemination, cervical cap insemination, and the like. The sperm may be added to the medium or the medium may be added to the sperm. Preferably, the medium contains gum guar, gum arabic, pectin or galacturonic acid, although another PCAGH may be used. In other aspects of this invention, methods are provided for the culture of such extended sperm to increase their survival during holding or culture at a range of temperatures from about room temperature (e.g., 20.degree. C.) to about body temperature (e.g., 37.degree. C. or 39.degree. C.). This includes culture of sperm in toxicity screen tests and

DEPR:

ORPL:

2 of 2

WEST☐ Generate Collection

L13: Entry 7 of 20

File: USPT

Jun 8, 1999

DOCUMENT-IDENTIFIER: US 5910568 A

TITLE: Molecule involved in binding of sperm to egg surfaces and procedures for use of this molecule to enhance or decrease potential fertility

ABPL:

A purified polypeptide which provides for initial binding of sperm to oocyte investments and has an active amino acid sequence of SEQ ID NO:12 (Cys-Gln-Ser-Leu-Gln-Glu-Tyr-Leu-Ala-Glu-Gln-Asn-Gln-Arg-Gln-Leu-Glu-Ser-A sn -Lys-Ile-Pro-Glu-Val-Asp-Leu-Ala-Arg-Val-Val-Ala-Pro-Phe-Met-Ser-Asn-Ile-P ro-Leu-Leu-Leu-Tyr-Pro-Gln-Asp-Arg-Pro -Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn-Glu-Asp-Val-Cys); or SEQ ID NO:13 (Cys-Glu-Ser-Leu-Gln-Lys-His-Leu-Ala-Glu-Leu-Asn-His-Gln-Lys-Gln-Leu-Glu-S er-Asn-Lys-Ile-Pro-Glu-Leu -Asp-Met-Thr-Glu-Val-Val-Ala-Pro-Phe-Met-Ala-Asn-Ile-Pro-Leu-Leu-Leu-Tyr-P ro-Gln-Asp-Gly-Pro-Arg-Ser-Lys-Pro-Gln -Pro-Lys-Asp-Asn-Gly-Asp-Val-Cys); or the shorter but biologically active SEQ ID NO:1 and SEQ ID NO:9 (Tyr-Pro-Gln-Asp-Arg-X-Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn, where X is Thr or Pro). The polypeptide is useful in improving sperm-egg binding by fresh sperm, restoring sperm-egg binding following a cryopreservation cycle, enhancing fertilizing potential, and producing antibodies to determine fertilizing potential of sperm, determining sperm-binding sites on egg investments, and contraception.

BSPR:

The present invention is a purified polypeptide (termed universal primary sperm-egg binding protein, or UPSEBP) which provides for initial bonding of sperm to oocyte investments and uses of the polypeptide. The inventive polypeptide has biological activity in a variety of avian and mammalian species and has active sites within the amino acid sequence embodied in SEQ ID NO:12 which is Cys-Gln-Ser-Leu-Gln-Glu-Tyr-Leu-Ala-Glu-Gln-Asn-Gln-Arg-Gln-Leu-Glu-Ser-As n-Lys-Ile-Pro-Glu-Val-Asp-Leu-Ala-Arg -Val-Val-Ala-Pro-Phe-Met-Ser-Asn-Ile-Pro-Leu-Leu-Leu-Tyr-Pro-Gln-Asp-Arg-P ro-Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn-Glu-Asp-Val-Cys. One of these active sites is within the portion of the amino acid sequence embodied in SEQ ID NO:9 or Tyr-Pro-Gln-Asp-Arg-X-Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn, where X is Thr or Pro. The tertiary structure of SEQ ID NO:12 or a similar sequence affects biological activity. Uses of the synthetic or natural polypeptide include in vitro treatment of sperm to restore fertilizing capacity for some samples of thawed cryopreserved sperm or to enhance fertilizing potential of some fresh sperm, intra-vaginal treatment of sperm to enhance sperm-egg binding and increase probability of fertilization, and use in an in vitro assay to determine fertilizing potential of sperm. Antibodies to the polypeptide are also useful for in vitro assays to determine potential fertility of sperm or number of sperm-binding sites on an egg investment or in vivo as a contraceptive.

DRPR:

FIG. 1 compares the number of sperm bound before and after cryopreservation, treated with UPSEBP and untreated to fresh sperm;

DRPR:

FIG. 7a shows the restorative capability of rooster UPSEBP (native protein

extracted from rooster sperm) and rat SGP-1 on binding of thawed cryopreserved turkey sperm;

DRPR:

FIG. 7b shows the restorative capability of turkey UPSEBP (native protein extracted from turkey sperm) on binding of thawed cryopreserved rooster sperm and thawed cryopreserved turkey sperm;

DRPR:

FIG. 9 shows the restorative capability of rooster UPSEBP (native protein extracted from rooster sperm) on binding of thawed cryopreserved stallion sperm.

DEPR:

Freshly collected rooster sperm were cryopreserved, thawed and processed to remove glycerol. One portion of the thawed sperm received crude UPSEBP at a concentration of 2 .mu.g protein per 10.sup.6 sperm. Samples containing 1-10.times.10.sup.6 sperm were placed into wells coated with an extract of hen's egg membrane and incubated 180 minutes. Non-bound sperm were removed by washing, and bound sperm were stained with diamido-2-phenylindole and counted using an epifluorescent microscope. The results presented in FIG. 1 demonstrate the ability of UPSEBP to restore the capability of frozen-thawed rooster sperm to bind to hen's egg membrane protein in comparison to unprocessed sperm.

DEPR:

Sperm in a sample of pooled semen were used to obtain fertility data before cryopreservation (i.e., fresh) and after cryopreservation and post-thaw processing to slowly remove glycerol (frozen-thawed). One portion of the thawed sperm was suspended in Minnesota-A buffer alone, and the second was treated with Minnesota-A buffer containing crude UPSEBP at 2 .mu.g protein per 10.sup.6 sperm. As shown in FIG. 4, fertility of the sperm processed in UPSEBP was double that for sperm not treated with UPSEBP and approached the level of fresh sperm. Hatchability of eggs artificially fertilized by sperm treated with UPSEBP increased from 18 to 25 percent.

DEPR:

Rooster sperm subjected to a cryopreservation cycle were treated with increasing amounts of partially purified UPSEBP and subject to a standard binding assay, together with aliquots of unfrozen (fresh) sperm. FIG. 6 shows that treatment of frozen-thawed rooster sperm with 0.04-4.0 .mu.g UPSEBP per 10.sup.6 sperm partially restored the sperm's capability to bind to the membrane of a hen's egg.

DEPR:

A primary antibody was produced in two rabbits against SEQ ID NO:5, linked via the cysteine amino acid to keyhole limpet, with use of standard adjuvants and techniques. The immune serum was harvested and used as a primary antibody to label rooster sperm, fixed in low ionic strength buffer using paraformaldehyde and processed by standard techniques, after which fluorescein isothiocyanate-labeled goat-antisheep gamma globulin was used as a second antibody. Analyses by flow cytometry revealed the percent of sperm as labeled, and visual examinations by epifluorescence microscopy revealed localization of the antibodies to the head region. A similar antibody was prepared against SEQ ID NO:12.

WEST

Generate Collection

L13: Entry 2 of 20

File: USPT

Aug 15, 2000

DOCUMENT-IDENTIFIER: US 6103483 A

TITLE: Molecule involved in binding of sperm to egg surfaces and procedures for use of this molecule to enhance or decrease potential fertility

ABPL:

A purified polypeptide which provides for initial binding of sperm to oocyte investments and has an active amino acid sequence of SEQ ID NO:12

(Cys-Gln-Ser-Leu-Gln-Glu-Tyr-Leu-Ala-Glu-Gln-Asn-Gln-Arg-Gln-Leu-Glu-Ser-Asn-Lys-Ile-Pro-Glu-Val-Asp-Leu-Ala-Arg-Val-Val-Ala-Pro-Phe-Met-Ser-Asn-Ile-Pro-Leu-Leu-Leu-Tyr-Pro-Gln-Asp-Arg-Pro-Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn-Glu-Asp-Val-Cys); or SEQ ID NO:13

(Cys-Glu-Ser-Leu-Gln-Lys-His-Leu-Ala-Glu-Leu-Asn-His-Gln-Lys-Gln-Leu-Glu-Ser-Asn-Lys-Ile-Pro-Glu-Leu-Asp-Met-Thr-Glu-Val-Val-Ala-Pro-Phe-Met-Ala-Asn-Ile-Pro-Leu-Leu-Leu-Tyr-Pro-Gln-Asp-Gly-Pro-Arg-Ser-Lys-Pro-Gln-Pro-Lys-Asp-Asn-Gly-Asp-Val-Cys); or the shorter but biologically active SEQ ID NO:1 and SEQ ID NO:9 (Tyr-Pro-Gln-Asp-Arg-X-Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn, where X is Thr or Pro). The polypeptide is useful in improving sperm-egg binding by fresh sperm, restoring sperm-egg binding following a cryopreservation cycle, enhancing fertilizing potential, and producing antibodies to determine fertilizing potential of sperm, determining sperm-binding sites on egg investments, and contraception.

BSPR:

include in vitro treatment of sperm to restore fertilizing capacity for some samples of thawed cryopreserved sperm or to enhance fertilizing potential of some fresh sperm, intra-vaginal treatment of sperm to enhance sperm-egg binding and increase probability of fertilization, and use in an in vitro assay to determine fertilizing potential of sperm. Antibodies to the polypeptide are also useful for in vitro assays to determine potential fertility of sperm or number of sperm-binding sites on an egg investment or in vivo as a contraceptive.

DRPR:

FIG. 1 compares the number of sperm bound before and after cryopreservation, treated with UPSEBP and untreated to fresh sperm;

DRPR:

FIG. 7a shows the restorative capability of rooster UPSEBP (native protein extracted from rooster sperm) and rat SGP-1 on binding of thawed cryopreserved turkey sperm;

DRPR:

FIG. 7b shows the restorative capability of turkey UPSEBP (native protein extracted from turkey sperm) on binding of thawed cryopreserved rooster sperm and thawed cryopreserved turkey sperm;

DRPR:

FIG. 9 shows the restorative capability of rooster UPSEBP (native protein extracted from rooster sperm) on binding of thawed cryopreserved stallion sperm.

DEPR:

Freshly collected rooster sperm were cryopreserved, thawed and processed to

remove glycerol. One portion of the thawed sperm received crude UPSEBP at a concentration of 2 .mu.g protein per 10.sup.6 sperm. Samples containing 1-10.times.10.sup.6 sperm were placed into wells coated with an extract of hen's egg membrane and incubated 180 minutes. Non-bound sperm were removed by washing, and bound sperm were stained with diamido-2-phenylindole and counted using an epifluorescent microscope. The results presented in FIG. 1 demonstrate the ability of UPSEBP to restore the capability of frozen-thawed rooster sperm to bind to hen's egg membrane protein in comparison to unprocessed sperm.

DEPR:

Sperm in a sample of pooled semen were used to obtain fertility data before cryopreservation (i.e., fresh) and after cryopreservation and post-thaw processing to slowly remove glycerol (frozen-thawed). One portion of the thawed sperm was suspended in Minnesota-A buffer alone, and the second was treated with Minnesota-A buffer containing crude UPSEBP at 2 .mu.g protein per 10.sup.6 sperm. As shown in FIG. 4, fertility of the sperm processed in UPSEBP was double that for sperm not treated with UPSEBP and approached the level of fresh sperm. Hatchability of eggs artificially fertilized by sperm treated with UPSEBP increased from 18 to 25 percent.

DEPR:

Rooster sperm subjected to a cryopreservation cycle were treated with increasing amounts of partially purified UPSEBP and subject to a standard binding assay, together with aliquots of unfrozen (fresh) sperm. FIG. 6 shows that treatment of frozen-thawed rooster sperm with 0.04-4.0 .mu.g UPSEBP per 10.sup.6 sperm partially restored the sperm's capability to bind to the membrane of a hen's egg.

DEPR:

A primary antibody was produced in two rabbits against SEQ ID NO:5, linked via the cysteine amino acid to keyhole limpet, with use of standard adjuvants and techniques. The immune serum was harvested and used as a primary antibody to label rooster sperm, fixed in low ionic strength buffer using paraformaldehyde and processed by standard techniques, after which fluorescein isothiocyanate-labeled goat-antisheep gamma globulin was used as a second antibody. Analyses by flow cytometry revealed the percent of sperm as labeled, and visual examinations by epifluorescence microscopy revealed localization of the antibodies to the head region. A similar antibody was prepared against SEQ ID NO:12.

WEST

Generate Collection

L13: Entry 17 of 20

File: USPT

Jan 21, 1997

DOCUMENT-IDENTIFIER: US 5595866 A

TITLE: Step-wise method to remove cryoprotectant from sperm

DEPR:

TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:

The addition of CPA to the sperm before cooling and its removal from sperm after warming are two of very important procedures in sperm cryopreservation. The present invention offers a new methodology to define optimal procedures to carry out either or both of these procedures in a fashion to reduce osmotic injury of the sperm. The calculated procedures were implemented and the results agree remarkably well with prediction.

DEPR:

Finally, it should be remembered that the effect of potential chemical toxicity of CPA on sperm cell viability is another reason causing sperm cell injury during the addition and removal of CPA for sperm cell cryopreservation. Given a CPA type and CPA concentration, it is generally accepted that the potential toxicity of the CPA to the cells is decreased with a decrease of cell exposure time to the CPA and a decrease in temperature.

WEST

Generate Collection

L13: Entry 13 of 20

File: USPT

Nov 25, 1997

DOCUMENT-IDENTIFIER: US 5691133 A

TITLE: Method to quickly add cryoprotectants to sperm cells while maintaining viability

DEPR:

TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:

The addition of CPA to the sperm before cooling and its removal from sperm after warming are two of very important procedures in sperm cryopreservation. The present invention offers a new methodology to define optimal procedures to carry out either or both of these procedures in a fashion to reduce osmotic injury of the sperm. The calculated procedures were implemented and the results agree remarkably well with prediction.

DEPR:

Finally, it should be remembered that the effect of potential chemical toxicity of CPA on sperm cell viability is another reason causing sperm cell injury during the addition and removal of CPA for sperm cell cryopreservation. Given a CPA type and CPA concentration, it is generally accepted that the potential toxicity of the CPA to the cells is decreased with a decrease of cell exposure time to the CPA and a decrease in temperature.

ORPL:

Acta Vet. Scand., vol. 32, No. 4, issued 1991, C.O. Bwanga, "Cryopreservation of Boar Sperm", pp. 431-453.

WEST

Generate Collection

L13: Entry 3 of 20

File: USPT

Apr 25, 2000

DOCUMENT-IDENTIFIER: US 6054287 A

TITLE: Cell-type-specific methods and devices for the low temperature preservation of the cells of an animal species

DEPR:

TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:

The addition of CPA to the sperm before cooling and its removal from sperm after warming are two of very important procedures in sperm cryopreservation. The present invention offers a new methodology to define optimal procedures to carry out either or both of these procedures in a fashion to reduce osmotic injury of the sperm. The calculated procedures were implemented and the results agree remarkably well with prediction.

DEPR:

Finally, it should be remembered that the effect of potential chemical toxicity of CPA on sperm cell viability is another reason causing sperm cell injury during the addition and removal of CPA for sperm cell cryopreservation. Given a CPA type and CPA concentration, it is generally accepted that the potential toxicity of the CPA to the cells is decreased with a decrease of cell exposure time to the CPA and a decrease in temperature.

WEST

Generate Collection

L13: Entry 11 of 20

File: USPT

May 19, 1998

DOCUMENT-IDENTIFIER: US 5753427 A

TITLE: General method to quickly add cryoprotectants to animal cells while maintaining viability

DEPR:

TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:

The addition of CPA to the sperm before cooling and its removal from sperm after warming are two of very important procedures in sperm cryopreservation. The present invention offers a new methodology to define optimal procedures to carry out either or both of these procedures in a fashion to reduce osmotic injury of the sperm. The calculated procedures were implemented and the results agree remarkably well with prediction.

DEPR:

Finally, it should be remembered that the effect of potential chemical toxicity of CPA on sperm cell viability is another reason causing sperm cell injury during the addition and removal of CPA for sperm cell cryopreservation. Given a CPA type and CPA concentration, it is generally accepted that the potential toxicity of the CPA to the cells is decreased with a decrease of cell exposure time to the CPA and a decrease in temperature.

ORPL:

Acta Vet. Scand., vol. 32, No. 4, issued 1991, C.O. Bwanga, "Cryopreservation of Boar Sperm", pp. 431-453.

WEST

Generate Collection

L13: Entry 9 of 20

File: USPT

Jul 7, 1998

DOCUMENT-IDENTIFIER: US 5776769 A

TITLE: Cell-type specific methods and devices for the low temperature preservation of the cells of an animal species

DEPR:

TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:

The addition of CPA to the sperm before cooling and its removal from sperm after warming are two of very important procedures in sperm cryopreservation. The present invention offers a new methodology to define optimal procedures to carry out either or both of these procedures in a fashion to reduce osmotic injury of the sperm. The calculated procedures were implemented and the results agree remarkably well with prediction.

DEPR:

Finally, it should be remembered that the effect of potential chemical toxicity of CPA on sperm cell viability is another reason causing sperm cell injury during the addition and removal of CPA for sperm cell cryopreservation. Given a CPA type and CPA concentration, it is generally accepted that the potential toxicity of the CPA to the cells is decreased with a decrease of cell exposure time to the CPA and a decrease in temperature.

ORPL:

Acta Vet. Scand., vol. 32, No. 4, issued 1991, C.O. Bwanga, "Cryopreservation of Boar Sperm", pp. 431-453.

Office Action Summary

Application No.
09/478,299

Applicant(s)
Schenk

Examiner
Michael Meller

Group Art Unit
1651



☒ Responsive to communication(s) filed on Nov 1, 2000

☐ This action is **FINAL**.

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 35 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire three month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claim

☒ Claim(s) 1-37 is/are pending in the application

Of the above, claim(s) 27-34, 36, and 37 is/are withdrawn from consideration

☐ Claim(s) _____ is/are allowed.

☒ Claim(s) 1-26 and 35 is/are rejected.

☐ Claim(s) _____ is/are objected to.

☐ Claims _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐ approved ☐ disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☒ None of the CERTIFIED copies of the priority documents have been
☐ received.

☐ received in Application No. (Series Code/Serial Number) _____.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____

☒ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☒ Notice of References Cited, PTO-892

☒ Information Disclosure Statement(s), PTO-1449, Paper No(s). 3

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

— SEE OFFICE ACTION ON THE FOLLOWING PAGES —

Art Unit: 1651

DETAILED ACTION

Election/Restriction

1. Applicant's election with traverse of the election of species requirement in Paper No. 7 is acknowledged. It is noted on the record that the applicant did not traverse the restriction requirement but did elect Group I, claims 1-26 and 35 in paper number 4. The traversal of the election of species is on the ground(s) that ten independent and distinct nucleotide sequences have been determined to be a "reasonable number" of sequences to be claimed and examined in a single application without restriction and that searching for more than one cryoprotectant and more than one additional component would not be unduly burdensome to the examiner. This is not found persuasive because of the extensive literature search that is involved in the searching of such components. Applicant is reminded that while the literature search is extensive it is not co-extensive.

Thus, claims 27-34, 36 and 37 remain withdrawn from further consideration by the examiner and the species that applicants have elected reads on the cryoprotectant glycerol and a component that maintains osmolality and buffers pH. It is noted that applicant has also elected from claim 15, but the election of species was directed only to an additional component from claim 12.

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The requirement is still deemed proper and is therefore made FINAL.

Claim Rejections - 35 USC § 112

2. The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

3. Claims 1-26 and 35 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

In claims 1 and 35, step c), which calls for “isolating sperm from said selected sperm sample to produce isolated sperm “, is confusing since a sperm sample is isolated once it is no longer in the testicles. Step c, is thus redundant in its recitation of “isolating sperm” since the sperm is already isolated.

Further, the claim is confusing since the term, “final extender “ is confusing. What is the final extender final to ? There is no beginning extender.

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Claim Rejections - 35 USC § 103

4. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

5. Claims 1-26 and 35 are rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. taken with Spaulding.

The claims are drawn to a method for the cryopreservation of sperm as in claims 1 and 35 using as an extender the elected extender containing glycerol and a component that maintains osmolality and buffers pH.

Salisbury teaches cryopreservation of bull semen using different extenders including glycerol (page 497) and a component that maintains osmolality and buffers pH such as sodium citrate, Tris, milk, etc. Salisbury also teaches obtaining the sperm sample cooling it to 5°C for 140 minutes (see page 475-see also pages 463-464 for additional cooling semen), adding the extender (see page 456, 499), and freezing the suspension of sperm (see pages 471, 494, 495, 503-504). The pH of the extender is 6.5 to 7.0 (page 502 and page 456). See entire reference especially the pages mentioned.

Salisbury does not teach to specifically isolate the sperm using centrifugation and does not teach using flow cytometry to select the sperm sample.

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Spaulding teaches to sort sperm for their X or Y characteristic, by flow cytometry and to centrifuge sperm cells to remove seminal plasma proteins, i.e. isolate the sperm, after cooling, see example I.

It would have been obvious to one of ordinary skill in the art at the time the invention was made to use flow cytometry to select sperm for a particular characteristic in the method of Salisbury since Spaulding teaches using flow cytometry to sort X from Y sperm. To centrifuge (isolate) the sperm sample of Salisbury after cooling would have been obvious since Spaulding teaches to remove seminal plasma proteins, i.e. isolate the sperm, after cooling.

Since Salisbury uses bull sperm it also would have been obvious to use other mammalian sperm such as equine and porcine since they are also mammalian sperm and would be expected to work well in the process of Salisbury as well. To use different mammalian sperm is well within the purview of the skilled artisan.

A 50% to 90% recovery of sperm from the centrifugation is obvious since one would want to yield as much of the sperm as possible.

The concentration of sperm in said suspension prior to freezing would be inherent to the suspension of sperm.

The order of the steps of the process of Salisbury might be out of order from the claimed process, but this still is obvious. MPEP 2144.04 IV (C) states that it is *prima facie* obvious to perform in any order steps of a process.

Thus, the claimed subject matter is obvious over the cited references.

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Any inquiries concerning this communication should be directed to Examiner Mike Meller at telephone number (703) 308-4230. If attempts to reach the examiner by telephone are unsuccessful, the Examiner's supervisor, Michael Wityshyn, can be reached at (703) 308-4743. The Fax phone number for the art unit is (703) 308- 0294. Any inquiries of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.



DAVID M. NAFF
PRIMARY EXAMINER
ART UNIT 1651

Notice of References CitedApplication No.
09/478,299Applicant(s)
SchenkExaminer
Michael MellerGroup Art Unit
1651

Page 1 of 1

U.S. PATENT DOCUMENTS

	DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS
A	5,021,244	6/1991	Spaulding	424	561
B					
C					
D					
E					
F					
G					
H					
I					
J					
K					
L					
M					

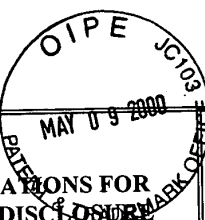
FOREIGN PATENT DOCUMENTS

	DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS
N						
O						
P						
Q						
R						
S						
T						

NON-PATENT DOCUMENTS

	DOCUMENT (Including Author, Title, Source, and Pertinent Pages)	DATE
U		
V		
W		
X		

FORM PTO-1449 (Modified)

ATTY. DOCKET NO.
22091-701 CON1SERIAL NO.
09/478,299LIST OF PATENTS AND PUBLICATIONS FOR
APPLICANT(S) INFORMATION DISCLOSURE
STATEMENT

(Use several sheets if necessary)

APPLICANT
John L. SchenkFILING DATE
January 5, 2000GROUP ART UNIT
~~1648~~ 1651

REFERENCE DESIGNATION

U.S. PATENT DOCUMENTS

EXAM'R INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	Subclass	Filing Date If Appropriate
JMS	A1	4,267,268	May 12, 1981	Nelson, Jr.	435	2	
	A2	4,980,277	December 25, 1990	Junnila	435	2	
	A3	5,135,759	August 4, 1992	Johnson	424	561	
	A4	5,514,537	May 7, 1996	Chandler	435	2	

FOREIGN PATENT DOCUMENTS

EXAM'R INITIAL		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	Subclass	TRANSLATION	
							yes	no
JMS	B1	WO 99/33956	July 8, 1999	PCT				

OTHER ART (Include Author, Title, Date, Pertinent Pages, etc.)

JMS	C1	Amann, Rupert P.; "Cryopreservation of Sperm," <i>Encyclopedia of Reproduction</i> , 1:733-783 (1999).
	C2	Arriola, J. and Foote, R. H.; "Glycerolation and Thawing Effects on Bull Spermatozoa Frozen in Detergent-Treated Egg Yolk and Whole Egg Extenders," <i>J Dairy Sci</i> , 70:1664-1670 (1987).
	C3	Behrman, S. J. and Ackerman, D. R.; "Freeze Preservation of Human Sperm," <i>Am. J. Obstet. and Gynec.</i> , 103:5:654-664 (1969).
	C4	Foote, R. H., "Buffers and Extenders: What Do They Do? Why Are They Important?" <i>Proc of the 10th NAAB Tech. Conf. on Artificial Insemination and Reproduction</i> , 62-70 (1984).
	C5	Graham, J. K. and Hammerstedt, R. H.; "Differential Effects of Butylated Hydroxytoluene Analogs on Bull Sperm Subjected to Cold-Induced Membrane Stress," <i>Cryobiology</i> , 29:106-117 (1992).
	C6	Hammerstedt, et al, "Cryopreservation of Mammalian Sperm: What We Ask Them to Survive," <i>Journal of Andrology</i> , 11:1:73-88 (1990).
	C7	Maxwell, W. M. C. and Johnson, L. A.; "Chlortetracycline Analysis of Boar Spermatozoa After Incubation, Flow Cytometric Sorting, Cooling, or Cryopreservation," <i>Molecular Reproduction and Development</i> , 46:408-418 (1997).
	C8	Polge, et al, "Revival of Spermatozoa After Vitrification and Dehydration at Low Temperatures," <i>Nature</i> , 164:666 (1949).

EXAMINER

DATE CONSIDERED

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant(s).

(Information Disclosure Statement -- Section 9 PTO-1449 (Modified) [6-1] Page 1 of 2

52018542.1

Docket No.: 22091-701CON1



Serial Number: 09/478,299

OTHER ART (Include Author, Title, Date, Pertinent Pages, etc.) [CONTINUED]

☒	C9	Pursel, et al, "Effect of Orvus ES Paste on Acrosome Morphology, Motility and Fertilizing Capacity of Frozen-Thawed Boar Sperm," <i>Journal of Animal Science</i> , 47:1:198-202 (1978).
☒	C10	Salisbury, et al, <i>Physiology of Reproduction and Artificial Insemination of Cattle</i> , 2nd Ed., San Francisco: W. H. Freeman, 442-554 (1978).
☒	C11	Schenk, et al, "Cryopreservation of Flow-Sorted Bovine Spermatozoa," <i>Theriogenology</i> , 52:1375-1391 (1999).
☒	C12	Seidel, et al, "Insemination of Heifers with Sexed Sperm," <i>Theriogenology</i> , 52:1407-1420 (1999).
☒	C13	Watson, "Recent Developments and Concepts in the Cryopreservation of Spermatozoa and the Assessment of Their Post-Thawing Function," <i>Reprod. Fertil. Dev.</i> , 7:871-891 (1995).

EXAMINER

DATE CONSIDERED

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant(s).

WEST**End of Result Set**

Generate Collection

L7: Entry 1 of 1

File: DWPI

DERWENT-ACC-NO: 1972-03539T
DERWENT-WEEK: 197203
COPYRIGHT 2001 DERWENT INFORMATION LTD

TITLE: Ovular and seminal cell extracts - having a haploid chromosome set for
therapeutic and diagnostic use

PATENT-ASSIGNEE:

ASSIGNEE

THEURER K

CODE

THE I

PRIORITY-DATA: 1970DE-2032988 (July 3, 1970)

PATENT-FAMILY:

PUB-NO

PUB-DATE

LANGUAGE

PAGES

MAIN-IPC

DE 2032988 A

N/A

000

N/A

INT-CL (IPC): A61K 17/00

ABSTRACTED-PUB-NO: DE 2032988A
BASIC-ABSTRACT:

Prepn. and use in therapy and diagnostic use and ovular cell extracts having a haploid chromosome set from corpuscular and high molecular fractions (nucleic acids, proteins, lipides and polysaccharides) and their degradation products, for aiding cell-proliferation and treating sterility and fertility. Antibody-sera against fractions with X- or Y-chromosomes can be used for sex-determination. Cells from fish are esp. suitable as starting materials. In example, for an antibody-serum against bovine Y-chromosomes cell-nuclei are obtained by grinding oval cells from trout-roe in liquid N₂, centrifuging in a saccharose-gradient, freeze-drying and decomposing chemically. Antibody serum from rabbits immunised with the oval product is incubated with bull-sperm. The non-agglutinated sperm-cells are isolated and the nuclei from the spermatozoa with Y-chromosomes used for the active immunisation of rabbits or sheep.

TITLE-TERMS: OVUM SEMEN CELL EXTRACT HAPLOID CHROMOSOME SET THERAPEUTIC
DIAGNOSE

DERWENT-CLASS: B04

CPI-CODES: B04-B02D; B04-B04A; B04-B04C; B12-G01; B12-G04; B12-J01; B12-K04;
B12-L05;

CHEMICAL-CODES:

Chemical Indexing M1 *01*

Fragmentation Code

V622 V624 V795 V751 V752 V753 V754 V600 V611 N160
P610 P620 M720 P710 P831 P832 M781 P930 Q252 R000
M422 M902

2/26/01 10:02 AM

WEST

Generate Collection

L12: Entry 2 of 3

File: USPT

Jun 6, 2000

DOCUMENT-IDENTIFIER: US 6071689 A

TITLE: System for improving yield of sexed embryos in mammals

ABPL:

Improved insemination systems particularly adapted to use for sex-selected sperm sorting include systems which achieve superovulation and then multiple embryo production with sexed embryos. These systems combine with other techniques, including techniques for enhanced sheath fluid and other strategies which minimize stress on the sperm cells, and, potentially, a 2.9 percent sodium citrate sheath solution for bovine species and a hepes bovine gamete media for equine species. Improved collection systems and techniques for the process are described so that commercial application of sperms samples as well as the resulting animals may be achieved.

BSPR:

For ages it has been desired to select the sex of specific offspring. Beyond obvious psychological aspects, the actual sex selection of mammalian offspring has significant economic consequences when one considers its application to food producing animals such as cattle as well as celebrated trophy animals such as horses and the like. This great desire has resulted in a significant variety of efforts to achieve sex-selected offspring. Probably the effort which has appeared most likely to achieve the desired results has been efforts at sorting and selecting between X and Y sperm prior to insemination.

DEPR:

Another aspect which may interplay in the various factors of the present invention is that of utilizing low dose amounts of sperm for artificial insemination or the like. Additional background on the aspect of sexed, artificial insemination may be found in "Prospects for Sorting Mammalian Sperm" by Rupert P. Amman and George E. Seidel, Jr., Colorado Associated University Press (1982) hereby incorporated by reference. As mentioned, natural insemination involves numbers of sperm on the order of billions of sperm. Typical artificial insemination is presently conducted with millions of sperm for bovine species and hundreds of millions of sperm for equine species. By the term "low dose" it is meant that the dosage of sperm utilized in the insemination event are less than one-half or preferably even less than about 10% of the typical number of sperm provided in a typical artificial insemination event. Thus, the term "low dose" is to be viewed in the context of the typical artificial insemination dosage or also as an absolute number. For bovine sperm where currently 1 to 10 million sperm are provided, a low dose process may be considered an absolute number of about 500,000 sperm or perhaps as low as 300,000 sperm or lower. In fact, through utilization of the techniques of the present invention, artificial insemination with good percentages of success has been shown with levels of insemination of sperm at 100,000 and 250,000 sperm (41% and 50%, respectively pregnancy rates). As shown in the article "Uterine Horn Insemination of Heifers With Very Low Numbers of Non-frozen and Sexed Spermatozoa" as published in 48 Theriogenology 1255 (1997) hereby incorporated by reference. Since sperm cells appear to display a sensitivity to dilution, these results may display particular interdependence on the utilization of low dose sperm samples with regards to various techniques of the present invention. The absolute numbers may be species dependent, for equine species, merely less than about ten, five, or even one million sperm may be considered a low dose process.

DEPR:

As examples, the following experiments have been conducted. While not all use every aspect of the inventions described here, they do show the performance enhancements possible through differing aspects of the invention. Further, a summary of some experiments is contained in the article "Uterine Horn Insemination of Heifers With Very Low Numbers of Non-frozen and Sexed Spermatozoa" as referenced earlier. This article summarizes some of the data showing the efficacy of the present invention. As to the experiments, one has been conducted with sexed, unfrozen sperm cells with high success as follows:

DEPR:

Angus heifers, 13-14 mo of age and in moderate body condition, were synchronized with 25 mg of prostaglandin F-2 alpha at 12-day intervals and inseminated 6-26 h after observed standing estrus. Freshly collected semen from three 14-26 mo old bulls was incubated in 38 .mu.M Hoechst 33342 at 75.times.10.sup.6 sperm/ml in a TALP medium for 1 h at 34.degree. C. Sperm were sorted by sex chromosomes on the basis of epifluorescence from laser excitation at 351 and 364 nm at 150 mW using a MoFlo.RTM. flow cytometer/cell sorter operating at 50 psi and using 2.9% Na citrate as sheath fluid. X chromosome-bearing sperm (.about.90% purity as verified by resorting sonicated sperm aliquots) were collected at .about.500 live sperm/sec into 2-ml Eppendorf tubes containing 100 .mu.l Cornell Universal Extender (CUE) with 20% egg yolk. Collected sperm were centrifuged at 600.times.g for 10 min and resuspended to 1.63.times.10.sup.6 live sperm/ml in CUE. For a liquid semen unsexed control; Hoechst 33342-stained sperm were diluted with sheath fluid to 9.times.10.sup.5 sperm/ml and centrifuged and resuspended to 1.63.times.10.sup.6 progressively motile sperm/ml in CUE. Sexed semen and liquid control semen were cooled to 5.degree. C. over 75 min and loaded into 0.25-ml straws (184 ul/straw). Straws were transported at 3 to 5.degree. C. in a temperature-controlled beverage cooler 240 km for insemination 5 to 9 h after sorting. Sexed semen and liquid control semen were inseminated using side-opening blue sheaths (IMV), one half of each straw into each uterine horn (3.times.10.sup.5 live sperm/heifer). As a standard control, semen from the same bulls had been frozen in 0.5-cc straws by standard procedures (mean 15.6.times.10.sup.6 motile sperm/dose post-thaw), thawed at 35.degree. C. for 30 sec, and inseminated into the uterine body. Treatments were balanced over the 3 bulls and 2 inseminators in a ratio of 3:2:2 inseminations for the sexed semen and two controls. Pregnancy was determined ultrasonically 31-34 days after insemination and confirmed 64-67 days later when fetuses also were sexed (blindly). Data are presented in the table.

DEPR:

The objective was to determine pregnancy rates when heifers are inseminated with extremely low numbers of frozen sperm under ideal field conditions. Semen from three Holstein bulls of above average fertility was extended in homogenized milk, 7% glycerol (CSS) extender plus 5% homologous seminal plasma to 2.times.10.sup.5, 5.times.10.sup.5 or 10.times.10.sup.6 (control) total sperm per 0.25 ml French straw and frozen in moving liquid nitrogen vapor. Semen was thawed in 37.degree. C. water for 20 sec. Holstein heifers 13-15 mo of age weighing 350-450 kg were injected with 25 mg prostaglandin F-2-alpha (Lutalyse.RTM.) twice at a 12-day interval and inseminated with an embryo transfer straw gun and side-opening sheath, half of the semen deep into each uterine horn 12 or 24 h after detection of estrus. The experiment was done in five replicates over 5 months, and balanced over two insemination technicians. Ambient temperature at breeding was frequently -10 to -20.degree. C., so care was taken to keep insemination equipment warm. Pregnancy was determined by detection of a viable fetus using ultrasound 40-44 days post-estrus and confirmed 55-62 days post-estrus; 4 of 202 conceptuses were lost between these times. Day 55-62 pregnancy rates were 55/103 (53%), 71/101, (70%), and 72/102 (71%) for 2.times.10.sup.5, 5.times.10.sup.5 and 10.times.10.sup.6 total sperm/inseminate (P<0.1). Pregnancy rates were different (P<0.05) among bulls (59, 62, and 74%), but not between technicians (64 and 65%) or inseminations times post-estrus (65% for 12 h and 64% for 24 h, N=153 at each time). With the methods described, pregnancy rates in heifers were similar with

5.times.10.sup.5 and 10.times.10.sup.6 total sperm per inseminate.

DEPL:

Although the pregnancy rate with sexed semen was only 80% of controls, this difference was not statistically significant (>0.1). One pregnancy was lost by 64-67 d in each of the sexed and frozen control groups; 18 of 19 fetuses (95%) were female in the sexed group, and 20 of 30 (67%) were female in the control groups. The liquid semen control yielded a virtually identical pregnancy rate to the frozen semen control containing over 50 times more motile sperm (over 120 times more total sperm), demonstrating the efficacy of low-dose insemination into the uterine horns. We have altered the sex ratio in cattle significantly using flow cytometer technology and artificial insemination.

DETL:

	No.	No.	Pregnant	No.	Pregnant	No female
fe- Treatment Heifers bred d31-34 d64-67 tuses						
(95%).sup.a Liquid	28	15	(54%)	15	(54%)	8 (53%).sup.b control
(55%) 15 (52%) 12 (80%).sup.c control						<u>Frozen</u> 29 16

.sup.a,b Sex ratios of values with different superscripts differ ($P < 0.02$).

WEST

Generate Collection

L12: Entry 1 of 3

File: USPT

Nov 21, 2000

DOCUMENT-IDENTIFIER: US 6149867 A

TITLE: Sheath fluids and collection systems for sex-specific cytometer sorting of sperm

ABPL:

Improved flow cytometer system particularly adapted to use for sex-selected sperm sorting include enhanced sheath fluid and other strategies which minimize stress on the sperm cells, including a 2.9 percent sodium citrate sheath solution for bovine species and a hepes bovine gamete media for equine species. Improved collection systems and techniques for the process are described so that commercial applications of sperms samples as well as the resulting animals may be achieved.

BSPR:

For ages it has been desired to select the sex of specific offspring. Beyond obvious psychological aspects, the actual sex selection of mammalian offspring has significant economic consequences when one considers its application to food producing animals such as cattle as well as celebrated trophy animals such as horses and the like. This great desire has resulted in a significant variety of efforts to achieve sex-selected offspring. Probably the effort which has appeared most likely to achieve the desired results has been efforts at sorting and selecting between X and Y sperm prior to insemination.

DEPR:

Another aspect which may interplay in the various factors of the present invention is that of utilizing low dose amounts of sperm for artificial insemination or the like. Additional background on the aspect of sexed, artificial insemination may be found in "Prospects for Sorting Mammalian Sperm" by Rupert P. Amman and George E. Seidel, Jr., Colorado Associated University Press (1982) hereby incorporated by reference. As mentioned, natural insemination involves numbers of sperm on the order of billions of sperm. Typical artificial insemination is presently conducted with millions of sperm for bovine species and hundreds of millions of sperm for equine species. By the term "low dose" it is meant that the dosage of sperm utilized in the insemination event are less than one-half or preferably even less than about 10% of the typical number of sperm provided in a typical artificial insemination event. Thus, the term "low dose" is to be viewed in the context of the typical artificial insemination dosage or also as an absolute number. For bovine sperm where currently 1 to 10 million sperm are provided, a low dose process may be considered an absolute number of about 500,000 sperm or perhaps as low as 300,000 sperm or lower. In fact, through utilization of the techniques of the present invention, artificial insemination with good percentages of success has been shown with levels of insemination of sperm at 100,000 and 250,000 sperm (41% and 50%, respectively pregnancy rates). As shown in the article "Uterine Horn Insemination of Heifers With Very Low Numbers of Non-frozen and Sexed Spermatozoa" as published in 48 Theriogenology 1255 (1997) hereby incorporated by reference. Since sperm cells appear to display a sensitivity to dilution, these results may display particular interdependence on the utilization of low dose sperm samples with regards to various techniques of the present invention. The absolute numbers may be species dependent, for equine species, merely less than about ten, five, or even one million sperm may be considered a low dose process.

DEPR:

As examples, the following experiments have been conducted. While not all use every aspect of the inventions described here, they do show the performance enhancements possible through differing aspects of the invention. Further, a summary of some experiments is contained in the article "Uterine Horn Insemination of Heifers With Very Low Numbers of Non-frozen and Sexed Spermatozoa" as referenced earlier. This article summarizes some of the data showing the efficacy of the present invention. As to the experiments, one has been conducted with sexed, unfrozen sperm cells with high success as follows:

DEPR:

Angus heifers, 13-14 mo of age and in moderate body condition, were synchronized with 25 mg of prostaglandin F-2 alpha at 12-day intervals and inseminated 6-26 h after observed standing estrus. Freshly collected semen from three 14-26 mo old bulls was incubated in 38 .mu.M Hoechst 33342 at 75.times.10.sup.6 sperm/ml in a TALP medium for 1 h at 34.degree. C. Sperm were sorted by sex chromosomes on the basis of epifluorescence from laser excitation at 351 and 364 nm at 150 mW using a MoFlo.RTM. flow cytometer/cell sorter operating at 50 psi and using 2.9% Na citrate as sheath fluid. X chromosome-bearing sperm (.about.90% purity as verified by resorting sonicated sperm aliquots) were collected at .about.500 live sperm/sec into 2-ml Eppendorf tubes containing 100 .mu.l Cornell Universal Extender (CUE) with 20% egg yolk. Collected sperm were centrifuged at 600.times.g for 10 min and resuspended to 1.63.times.10.sup.6 live sperm/ml in CUE. For a liquid semen unsexed control; Hoechst 33342-stained sperm were diluted with sheath fluid to 9.times.10.sup.5 sperm/ml and centrifuged and resuspended to 1.63.times.10.sup.6 progressively motile sperm/ml in CUE. Sexed semen and liquid control semen were cooled to 5.degree. C. over 75 min and loaded into 0.25-ml straws (184 ul/straw). Straws were transported at 3 to 5.degree. C. in a temperature-controlled beverage cooler 240 km for insemination 5 to 9 h after sorting. Sexed semen and liquid control semen were inseminated using side-opening blue sheaths (IMV), one half of each straw into each uterine horn (3.times.10.sup.5 live sperm/heifer). As a standard control, semen from the same bulls had been frozen in 0.5-cc straws by standard procedures (mean 15.6.times.10.sup.6 motile sperm/dose post-thaw), thawed at 35.degree. C. for 30 sec, and inseminated into the uterine body. Treatments were balanced over the 3 bulls and 2 inseminators in a ratio of 3:2:2 inseminations for the sexed semen and two controls. Pregnancy was determined ultrasonically 31-34 days after insemination and confirmed 64-67 days later when fetuses also were sexed (blindly). Data are presented in the table.

DEPR:

Although the pregnancy rate with sexed semen was only 80% of controls, this difference was not statistically significant (>0.1). One pregnancy was lost by 64-67d in each of the sexed and frozen control groups; 18 of 19 fetuses (95%) were female in the sexed group, and 20 of 30 (67%) were female in the control groups. The liquid semen control yielded a virtually identical pregnancy rate to the frozen semen control containing over 50 times more motile sperm (over 120 times more total sperm), demonstrating the efficacy of low-dose insemination into the uterine horns. We have altered the sex ratio in cattle significantly using flow cytometer technology and artificial insemination.

DEPR:

The objective was to determine pregnancy rates when heifers are inseminated with extremely low numbers of frozen sperm under ideal field conditions. Semen from three Holstein bulls of above average fertility was extended in homogenized milk, 7% glycerol (CSS) extender plus 5% homologous seminal plasma to 2.times.10.sup.5, 5.times.10.sup.5 or 10.times.10.sup.6 (control) total sperm per 0.25 ml French straw and frozen in moving liquid nitrogen vapor. Semen was thawed in 37.degree. C. water for 20 sec. Holstein heifers 13-15 mo of age weighing 350-450 kg were injected with 25 mg prostaglandin F-2-alpha (Lutalyse.RTM.) twice at a 12-day interval and inseminated with an embryo transfer straw gun and side-opening sheath, half of the semen deep into each uterine horn 12 or 24 h after detection of estrus. The experiment was done in five replicates over 5 months, and balanced over two insemination technicians.

Ambient temperature at breeding was frequently -10 to -20.degree. C., so care was taken to keep insemination equipment warm. Pregnancy was determined by detection of a viable fetus using ultrasound 40-44 days post-estrus and confirmed 55-62 days post-estrus; 4 of 202 conceptuses were lost between these times. Day 55-62 pregnancy rates were 55/103 (53%), 71/101, (70%), and 72/102 (71%) for 2.times.10.sup.5, 5.times.10.sup.5 and 10.times.10.sup.6 total sperm/inseminate (P<0.1). Pregnancy rates were different (P<0.05) among bulls (59, 62, and 74%), but not between technicians (64 and 65%) or inseminations times post-estrus (65% for 12 h and 64% for 24 h, N=153 at each time). With the methods described, pregnancy rates in heifers were similar with 5.times.10.sup.5 and 10.times.10.sup.6 total sperm per inseminate.

DETL:

No female Treatment bred d31-34 d64-67	No. Heifers	No. Pregnant	No. Pregnant
	fetuses		
	Sexed semen	45 20 (44%) 19 (42%) 18	
(95%).sup.a Liquid control 28 15 (54%) 15 (54%) 8 (53%).sup.b <u>Frozen</u> control 29			
16 (55%) 15 (52%) 12 (80%).sup.c			

.sup.a,b Sex ratios of values with different superscripts differ (P < 0.02).

ORPL:

"Insemination Of Heifers With Very Low Numbers Of Frozen Spermatozoa." G.E. Seidel, Jr., C.H. Allen, Z. Brink, M.D. Holland, and M.B. Cattell, Colorado State University, Fort Collins, Atlantic Breeders Cooperative, Lancaster, PA, DUO Dairy, Loveland, CO, Jul. 1996.

WEST**End of Result Set**

Generate Collection

L14: Entry 3 of 3

File: USPT

Dec 17, 1985

DOCUMENT-IDENTIFIER: US 4559309 A

TITLE: Flow cytometry-fluorescence measurements for characterizing sperm

BSPR:

To quantitate RNA content and DNA content/chromatin condensation a fresh semen sample (1-6 hours post emission) or frozen sample (allowing samples to be accumulated and sent to a laboratory) is treated with a detergent solution, stained with acridine orange (AO) and measured by flow cytometry (FCM); approximately ten minutes are required to measure 5,000 cells per sample and analyze the data with computer assistance. The following can be learned from a single measurement: (a) the percentage of each cell type in semen including, (i) mature sperm, (ii) immature sperm precursor cells representing all stages of development from spermatogonia to mature sperm, (iii) somatic cells, e.g. leukocytes; (b) normality/abnormality of sperm nuclear chromatin condensation. These measurements can be correlated with cell types in testis biopsies identified by two parameter FCM measurements (RNA vs. DNA) using acridine orange as the fluorescent probe and measuring simultaneously the red and the green fluorescent values of each cell and storing in computer memory the integrated values for each cell.

DEPR:

To quantitate RNA content and DNA content/chromatin condensation for each of many possible cell types and differentiation levels of the cells present in human semen, a fresh semen sample (1-6 hours post emission) or frozen sample (allowing samples to be accumulated and sent to a laboratory) was treated with a detergent solution, stained with acridine orange (AO) and measured by flow cytometry (FCM); approximately ten minutes are required to measure 5,000 cells per sample and analyze the data with computer assistance. The following can be learned from a single measurement: (a) the percentage of each cell type in semen including, (i) mature sperm, (ii) immature sperm precursor cells representing all stages of development from spermatogonia to mature sperm, (iii) somatic cells, e.g. leukocytes; (b) normality/abnormality of sperm nuclear chromatin condensation. These measurements were correlated with cell types in testis biopsies identified by two parameter FCM measurements (RNA vs. DNA) using acridine orange as the fluorescent probe being available for AO intercalation and any increase in red/red pulse green fluorescence is due to AO interaction with single strand DNA.

WEST

Generate Collection

L14: Entry 2 of 3

File: USPT

May 7, 1996

DOCUMENT-IDENTIFIER: US 5514537 A

TITLE: Process and apparatus for sorting spermatozoa

BSPR:

Certain dyes such as ethidium bromide and acridine orange have been used to evaluate DNA content of spermatozoa. Fluorescence intensity of the dye is proportional to the amount of dye absorbed. B. L. Gledhill et al., "Identifying X- and Y-Chromosome-Bearing Sperm by DNA Content: Retrospective Perspectives and Prospective Opinions," in R. P. Amann et al. (eds.) Prospects for Sexing Mammalian Sperm, pp. 177-91 (1982), discloses the use of 4',6-diamidino-2-phenylindole (DAPI) staining, and epi-illumination with orienting-flow cytometry, to show that the mean DNA content of bovine spermatozoa from frozen semen exhibited two distinct, but overlapping peaks of fluorescence. These peaks were nearly equal in size, and were separated by a 3.9% difference in intensity.



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JUN 22 2001

TECH CENTER 1600/2900

Docket No. 22091-701CON1

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a)):

[X] Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington D.C. 20231.

[] Pursuant to 37 C.F.R. § 1.6(d), I hereby certify that this paper and all enclosures are being sent via facsimile on the date indicated below to the attention of Examiner _____ at Facsimile No. _____ at _____ a.m./p.m.

Dated: June 13, 2001

Name of Person Certifying: Nancy L. Hug

Printed Name: Nancy L. Hug

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John L. Schenk

Filing Date: 01/05/2000

Serial No.: 09/478,299

Title: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

Assignee: XY, Inc.

Examiner: M. Meller

Group Art Unit: 1651

Assistant Commissioner for Patents

Washington, D.C. 20231

INFORMATION DISCLOSURE STATEMENT

Sir:

In accordance with 37 C.F.R. § 1.56, the references listed on the attached Form PTO-1449 are being brought to the attention of the Examiner for consideration in connection with the examination of the above-identified patent application.

I. Timing of the Information Disclosure Statement:

This Information Disclosure Statement is filed:

- ☐ With the new patent application submitted herewith (37 C.F.R. § 1.97(a)).
- ☐ Within three months after the filing date of the application or within three months after the date of entry of the national stage of a PCT application as set forth in 37 C.F.R. § 1.491.
- ☐ Before the mailing date of a first Office action on the merits. In the event, however, that an Office Action has crossed in the mail with this Information Disclosure Statement, the Commissioner is hereby authorized to charge Deposit Account No. [] for any fees required pursuant to 37 C.F.R. §§ 1.17(p) or 1.17(i)(1).



Docket No. 22091-701CON1

This Information Disclosure Statement is filed:

- ☐ After the first Office Action and more than three months after the application's filing date; or PCT national stage date of entry filing but, as far as is known to the undersigned, prior to the mailing date of either a final rejection or a notice of allowance, whichever occurs first, and the Commissioner is hereby authorized to charge Deposit Account No. [] for the fee (\$180) set forth in 37 C.F.R. § 1.17(p) and any additional required fees.

This Information Disclosure Statement is filed:

- ☐ After the mailing date of either a final rejection or a notice of allowance, whichever occurred first, and is accompanied by the fee (\$180.00) set forth in 37 C.F.R. § 1.17(i)(1) and a certification as specified in 37 C.F.R. § 1.97(e), as checked below. This document is to be considered as a petition requesting consideration of the Information Disclosure Statement.

The undersigned certifies that:

- ☒ Each item of information contained in the Information Disclosure Statement was the first citation of that item by a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this information disclosure statement.
- ☐ No item of information contained in this information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application or, to the knowledge of the undersigned after making reasonable inquiry, was known to any individual designated in 37 C.F.R. § 1.56(c) more than three months prior to the filing of this Information Disclosure Statement.

II. Copies of the Cited Items:

- ☒ Copies of all of the items listed on the attached Form PTO-1449 are enclosed.
- ☐ Copies of only the following items listed on the attached Form PTO-1449 are enclosed: _____.
- ☐ Copies of those items which are marked with an asterisk (*) in the attached Form PTO-1499 are not supplied because they were previously cited by or submitted to the Patent Office in a prior Application No. _____, filed _____ and relied upon in this application for an earlier filing date under 35 U.S.C § 120. See 37 C.F.R. § 1.98(d).



Docket No. 22091-701CON1

A copy of the search report and the cited references not already of record in this application are attached hereto.

III. Concise Explanation of Relevance:

- ☒ A concise explanation of relevance of the items listed on Form PTO-1449 is not given.
- ☐ A concise explanation of relevance of [some of] the items listed on Form PTO-1449 is in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references (copy attached).

IV. Conclusion:

Citation of the above documents shall not be construed as:

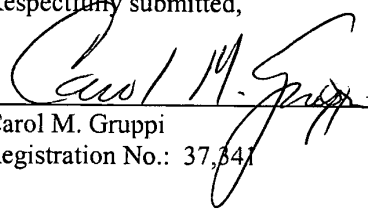
1. an admission that the documents are necessarily prior art with respect to the instant invention;
2. a representation that a search has been made, other than as described above; or
3. an admission that the information cited herein is, or is considered to be, material to patentability as defined in § 1.56(b).

It is respectfully requested that the Examiner indicate consideration of the cited references by returning a copy of the attached form PTO 1449 with initials or other appropriate marks.

The Commissioner is hereby authorized to charge Deposit Account No. 50-1189 Docket No.: 22091-701 for any additional fees required in connection with the filing of this Information Disclosure Statement.

DATE: June 13, 2001

Respectfully submitted,

By: 

Carol M. Gruppi

Registration No.: 37,341

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (415) 393-2000
Telefax: (415) 393-2286



Docket No.: 22091-701CON1

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a))
I hereby certify that this correspondence is, on the date shown below, being:

☒ MAILING deposited with the United States Postal Service as First Class Mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.	☐ FACSIMILE transmitted by facsimile to the Patent and Trademark Office. Name of Person Certifying: Carol M. Gruppi

Date: June 19, 2001

#11
M.G.J.
7/5/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE **RECEIVED**

Inventor(s): John Schenk

Assignee: XY, Inc.

JUN 29 2001

Serial No.: 09/478,299

Examiner: M. Meller

TECH CENTER 1600/2900

Filing Date: January 5, 2000

Group Art Unit: 1651

Title: Method of Cryopreserving Selected Sperm Cells

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

RESPONSE UNDER 37 C.F.R. § 1.111

Sir:

This is in response to the Office Action dated March 19, 2001. This response is accompanied by a petition for an extension of time to extend the period to respond to the Office Action to June 19, 2001, and the appropriate fee under 37 C.F.R. § 1.16 and § 1.17.

REMARKS

Claims 1-37 are pending in this application. Claims 27-34, 36 and 37 have been withdrawn from consideration. Claims 1-26 and 35 stand rejected. Reconsideration of the application in view of the following remarks is respectfully requested.

REJECTIONS UNDER 35 U.S.C. § 112, SECOND PARAGRAPH

Claims 1-26 and 35 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Examiner contends that the claims are indefinite due to the recitation of the phrases "isolating sperm from said selected sperm sample to produce isolated sperm" and "final extender". Applicant traverses this rejection for the reasons set forth below.

One of skill in the art would know that sperm cells no longer in the testicles are not "isolated" but accompanied by seminal fluid, proteins etc. Thus, Applicant submit that one of skill in the art would understand the recitation "isolating sperm from said selected sperm sample to produce isolated sperm". Accordingly, withdrawal of this rejection is respectfully requested. Likewise the phrase "final extender" is defined in the specification at page 4, lines 20-21. Thus, one of skill in the art will understand the recitation "final extender." Withdrawal of this ground of rejection is also respectfully requested.

REJECTIONS UNDER 35 U.S.C. § 103(a)

Claims 1-26 and 35 also stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Salisbury et al. taken with Spaulding (U.S. Patent No. 5, 012,244). Applicant respectfully traverses this rejection for the reasons set forth below.

The instantly claimed invention is directed to a method for freezing sperm selected for a particular characteristic (e.g., sex selected) which, when the sperm is thawed results in viable sperm that can be used in procedures such as artificial insemination ("AI") and in vitro fertilization ("IVF"). The method facilitates the storage and/or shipment of selected sperm samples to sites distant from the collection site and represents an important advance in livestock management, where selection of sperm for use in such procedures can be used to increase the production of offspring having desirable traits (see specification, page 3, lines 9-24).

Prior researchers had demonstrated that the stresses associated with various selection methods or with cryopreservation resulted in significant losses in fertility and/or viability of sperm. The selection and subsequent processing of sperm presents unique challenges because sperm is relatively susceptible to physical injury, are incapable of DNA repair and exhibit

decreased fertility with increasing time between collection and use. As most of the available selection methods involve physical stresses and take time, selected sperm are compromised compared to non-selected sperm. Fertility is generally further reduced if the selection technique (e.g., flow cytometry) involves significant dilution (see specification, page 1, lines 28-31, page 2, lines 1-5). In addition, the freezing of sperm invariably reduces fertility, motility, and/or viability (see specification, page 2, lines 15-17). Although techniques for freezing unselected sperm are known, *no technique for repeatedly producing viable cryopreserved selected sperm has been described prior to the instant disclosure.*

Moreover, prior to applicant's invention, no viable pregnancies had been achieved or no offspring exhibiting the selected characteristic had been produced using selected cryopreserved sperm. Thus, for one of ordinary skill in the art, there would be no reasonable expectation of success in using cryopreserved selected sperm to produce pregnancies which resulted in offspring with the selected characteristic. *The present inventors have demonstrated, for the first time, that pregnancies can be achieved with sperm that have been selected and then frozen and that the resulting offspring exhibit the selected characteristic.*

Salisbury is a general review relating to artificial insemination in cattle. Salisbury discusses extenders and the extension of unfrozen semen and some techniques for freezing unselected sperm. Salisbury does not teach or suggest that sperm selected for a particular characteristic may be frozen or that pregnancies can be achieved with such selected cryopreserved sperm. Such a disclosure is found only in the instant application. Thus, Salisbury cannot render the claimed invention obvious.

Spaulding relates to a method for sex selecting sperm utilizing sex associated membrane antibodies. Spaulding does not teach or suggest that sperm selected for a particular characteristic may be frozen and that pregnancies can be achieved with such selected cryopreserved sperm. Accordingly, Spaulding does not remedy the deficiency of Salisbury. Thus Spaulding, either alone or in combination can not render the claimed invention obvious.

Applicant respectfully submits that a proper analysis under 35 USC § 103 requires a consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success. Both the suggestion and

the reasonable expectation of success must be found in the prior art, not applicant's disclosure. In re Vaeck, 947 Fed. 2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). The cryopreservation of selected sperm and the use of such sperm to achieve viable pregnancies which result in offspring with the selected characteristic is found only in the instant application, and not in the cited references. Thus the cited references cannot render the claimed invention obvious. Accordingly, applicant respectfully requests withdrawal of this ground of rejection.

The Examiner also contends that while "the steps of the process of Salisbury might be out of order from the claimed process" the invention is still obvious because "it is prima facie obvious to perform in any order steps of a process" (MPEP 2144.04 IV (C)). Applicant respectfully traverse this rejection for the reasons discussed below.

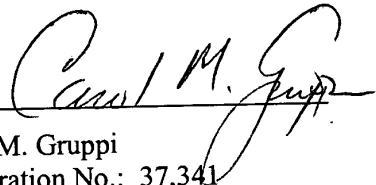
There is no basis in the law for treating combinations of old elements differently in determining patentability. Fromson v Advance Offset Plate, Inc., 755 F.2d 1549, 225 USPQ 26 (Fed. Cir. 1985). Thus, whether the combination of elements is old or new, an analysis under 35 USC § 103 requires consideration of the two factors discussed herein above. As also discussed above, to one of ordinary skill in the art, there would be no reasonable expectation of success in using cryopreserved selected sperm to produce pregnancies which resulted in offspring with the selected characteristic. Accordingly, the claimed invention is not rendered obvious and withdrawal of this ground of rejection is respectfully requested.

CONCLUSION

Applicants respectfully submit that the claims comply with 35 U.S.C. § 112, second paragraph and define an invention that is patentable over the art. Accordingly, allowance is in order, and an early notification to that effect would be appreciated. Should the Examiner in reviewing the communication have any questions or need any additional information, he is welcome to contact the undersigned at (650) 849-4902.

A fee of \$445.00 is due under 37 C.F.R. § 1.16 and § 1.17 for the Petition for a an Extension of Time filed concurrently herewith. The Assistant Commissioner is hereby authorized to charge any additional fees which may be required by this paper, or credit any overpayment to Deposit Account No. 50-1189. Docket No.: 22091-701CON1.

Respectfully submitted,

By: 

Carol M. Gruppi
Registration No.: 37,341

Dated: June 19, 2001

Mailing Address:

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (650) 849-4400
Telefax: (650) 849-4800



Docket No. 22091-701CON1

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Dated: June 19, 2001

Name of Person Certifying: _____
Printed Name: Carol M. Gruppi

Carol M. Gruppi

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JUN 29 2001

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#10
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7/5/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John L. Schenk

Serial No.: 09/478,299

Filing Date: 01/05/2000

Title: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

Assignee: XY, Inc.

Examiner: M. Meller

Group Art Unit: 1651

Assistant Commissioner for Patents
Washington, D.C. 20231

PETITION AND FEE FOR EXTENSION OF TIME
(37 C.F.R. § 1.136(a))

Sir:

Pursuant to 37 C.F.R. § 1.136(a), Applicant hereby petitions for a 3 month extension of time to respond to the Office Action mailed on December 19, 2000.

1. This communication is in connection with the matter for which this extension is requested

- a. ☒ is filed herewith; or
b. ☐ has been filed on _____.

2. ☒ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.

06/27/2001 BSAYAS11 00000030 501109 09478299

01 FC:217 445.00 CM

3. The following fees are submitted:

	TOTAL MONTHS REQUESTED	OTHER THAN SMALL ENTITY	SMALL ENTITY	CALCULATIONS
a. ☐	one month	\$110.00	\$55.00	\$
b. ☐	two month	\$390.00	\$195.00	\$
c. ☒	three month	\$890.00	\$445.00	\$445.00
d. ☐	four month	\$1,390.00	\$695.00	\$
e. ☐	five month	\$1,890.00	\$945.00	\$
f. ☐	An extension for ____ months has already been secured for filing the above-identified communication and the fee paid therefor of \$ ____ is deducted from the total fee due for the total months of extension now requested. The fee for this extension (\$ ____), minus the fee previously paid (\$ ____) equals \$ ____ (total fee due).			\$
TOTAL FEES =				\$445.00

- ☒ **Conditional Petition for Extension of Time:** An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701CON1, in the amount of \$445.00 to cover the above-fees. *A duplicate copy of this sheet is enclosed.*
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701CON1. *A duplicate copy of this sheet is enclosed.*

DATE: June 19, 2001

Respectfully submitted,

By: _____

Carol M. Gruppi

Registration No.: 37,341

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (415) 393-2000
Telefax: (415) 393-2286



\$ 1651

Docket No. 22091-701CON1

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a)):

[X] Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington D.C. 20231.

[] Pursuant to 37 C.F.R. § 1.6(d), I hereby certify that this paper and all enclosures are being sent via facsimile on the date indicated below to the attention of Examiner _____ at Facsimile No. _____ at _____ a.m./p.m.

Dated: June 19, 2001

Name of Person Certifying: Carol M. Grupp

Printed Name: Carol M. Grupp

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John L. Schenk

Assignee: XY, Inc.

Serial No.: 09/478,299

Examiner: M. Meller

Filing Date: 01/05/2000

Group Art Unit: 1651

Title: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

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JUN 29 2001

Assistant Commissioner for Patents
Washington, D.C. 20231

TECH CENTER 1600/2

RESPONSE & FEE TRANSMITTAL

Sir:

In response to the Office Action mailed on December 19, 2000, enclosed herewith for filing are the following:

- ☒ A Response [5] page(s)
- ☐ A Response to Restriction Requirement under 35 USC § 121 [] page(s)
- ☐ An Amendment Under 37 CFR § 1.111 [] page(s)
- ☐ An Amendment Under 37 CFR § 1.116 [] page(s)
- ☐ Other _____ [] page(s)

Also included are:

- ☒ A Petition for Extension of Time [3] months [2] page(s)
- ☐ Information Disclosure Statement
[] page(s) of PTO-1449 [] copies of IDS citations
- ☐ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.
- ☐ Other: _____
- ☒ Return Postcard

Fee Calculation						CALCULATIONS
☒ The following fees are submitted:						
EXTRA CLAIMS FEE				OTHER THAN SMALL ENTITY	SMALL ENTITY	
CLAIMS	CURRENT #	# OF CLAIMS PREVIOUSLY PAID	# EXTRA	RATE	RATE	
Total Claims	37-	37	0	× \$18.00	× \$9.00	\$0
Independent claims	3 -	3	0	× \$80.00	× \$40.00	\$0
MULTIPLE DEPENDENT CLAIM(S)						
☐ Yes ☐ No				\$270.00	\$135.00	\$
Petition for Extension of Time Fee (3_ months)						\$445.00
OTHER FEES _____ (specify)						\$
TOTAL FEES =						\$445.00

☒ Conditional Petition for Extension of Time: An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.

☐ A check in the amount of \$ _____ to cover the above fees is enclosed.

☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701CON1, in the amount of \$445.00 to cover the above-fees. *A duplicate copy of this sheet is enclosed.*

☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701CON1. *A duplicate copy of this sheet is enclosed.*

DATE: June 19, 2001

Respectfully submitted,

By: _____

Carol M. Gruppi

Registration No.: 37,341

McCutchen, Doyle, Brown & Enersen, LLP
 Three Embarcadero Center
 San Francisco, California 94111
 Telephone: (415) 393-2000
 Telefax: (415) 393-2286



UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

72

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.

09/478,299 01/05/00 SCHENK

J 22091-701

Emily M. Haliday
McCutchen Doyle Brown & Enersen LLP
Three Embarcadero Center
San Francisco CA 94111-4066

HM22/0907

EXAMINER

MELLER, M

ART UNIT

PAPER NUMBER

1651

DATE MAILED:

12

09/07/01

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Office Action Summary

Application No.

09/478,299

Applicant(s)

SCHENK, JOHN L.

Examiner

Michael V. Meller

Art Unit

1651

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 25 June 2001.
- 2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-37 is/are pending in the application.
- 4a) Of the above claim(s) 27-34, 36 and 37 is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 1-26 and 35 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
- Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
- 11) ☐ The proposed drawing correction filed on _____ is: a) ☐ approved b) ☐ disapproved by the Examiner.
- If approved, corrected drawings are required in reply to this Office action.
- 12) ☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

- 13) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
- * See the attached detailed Office action for a list of the certified copies not received.
- 14) ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).
- a) ☐ The translation of the foreign language provisional application has been received.
- 15) ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

- 1) ☐ Notice of References Cited (PTO-892)
- 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) ☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s) 9.
- 4) ☐ Interview Summary (PTO-413) Paper No(s) _____.
- 5) ☐ Notice of Informal Patent Application (PTO-152)
- 6) ☐ Other:

DETAILED ACTION

Election/Restrictions

The restriction requirement of record is maintained.

This application contains claims 27-34, 36 and 37 which are drawn to an invention nonelected with traverse in Paper No. 7. A complete reply to the final rejection must include cancelation of nonelected claims or other appropriate action (37 CFR 1.144) See MPEP § 821.01.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claim Rejections - 35 USC § 103

Claims 1-26 and 35 are rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. taken with Spaulding.

Applicants argue that Salisbury does not teach or suggest that sperm selected for a particular characteristic may be frozen or that pregnancies can be achieved with such selected cryopreserved sperm.

In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986).

Applicant cannot expect Salisbury to contain all of the elements of the claims since Salisbury was used in a 35 USC 103 rejection taken with Spaulding.

Further, Salisbury does teach freezing of the sperm and Spaulding teaches the selection of the sperm for a particular characteristic, namely, sex.

Also, the elected claims are not directed to pregnancies resulting from the use of the sperm, thus this argument by applicant is moot.

Thus, the claimed subject matter is properly rejected under this section.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any

Application/Control Number: 09/478,299
Art Unit: 1651

Page 4

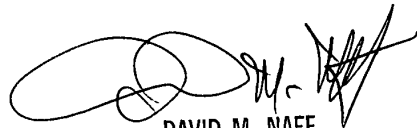
extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 703-308-4230. The examiner can normally be reached on Monday thru Friday: 10:30am-7:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Michael Wityshyn can be reached on 703-308-4743. The fax phone numbers for the organization where this application or proceeding is assigned are 703-308-0294 for regular communications and 703-308-0294 for After Final communications.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-0196.

MVM
September 6, 2001


DAVID M. NAFF
PRIMARY EXAMINER
ART UNIT 1651

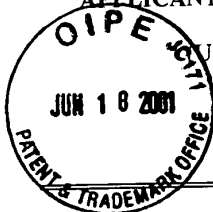
RECEIVED

JUN 22 2001

FORM PTO-1449 (Modified [6-1])

ATTY. DOCKET NO.
22091-0701CON1SERIAL NO.
TECH CENTER 1600/2900LIST OF PATENTS AND PUBLICATIONS FOR
APPLICANT(S)' INFORMATION DISCLOSURE
STATEMENT

Use several sheets if necessary)

INVENTOR
John L. SchenkFILING DATE
01/05/2000GROUP ART UNIT
1651

REFERENCE DESIGNATION

U.S. PATENT DOCUMENTS

EXAM'R INITIAL		DOCUMENT NUMBER	DATE	NAME	Class	Subclass	Filing Date If Appropriate
<i>mm</i>	A1	4,474,875	10/2/84	Shrimpton ~~et al~~	435	2	
	A2						
	A3						

FOREIGN PATENT DOCUMENTS

EXAM'R INITIAL		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	Subclass	TRANSLAT'N	
							yes	no
	B1							
	B2							
	B3							
	B4							
	B5							

OTHER ART (Include Author, Title, Date, Pertinent Pages, etc.)

<i>mm</i>	C1	Database WPI, Section CH, Week 199920; Derwent Publications Ltd., London, GB; Class B04, AN 1999-229816; XP002164167 & BR 9704313 (Alves EW, De Resende Matta M F et al (April 6, 1999 - Abstract).
	C2	DeLeeuw, F E et al. "Effects Of Various Cryoprotective Agents And Membrane-Stabilizing Compounds On Bull Sperm Membrane Integrity After Cooling And Freezing," (1993), <i>Cryobiology</i> , US, Academic Press Inc.; Vol. 30, No. 1, pp. 32-44, XP002089949; ISSN: 0011-2240.
	C3	Stap, J. et al, "Improving The Resolution Of Cryopreserved X- and Y-Sperm During DNA Flow Cytometric Analysis" (1998), <i>J. Anim. Sci.</i> ; Vol. 76, pp. 1896-1902, XP000985715
	C4	

EXAMINER

DATE CONSIDERED

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant(s).

Interview Summary	Application No.	Applicant(s)	
	09/478,299	SCHENK, JOHN L.	
	Examiner	Art Unit	
	Michael V. Meller	1651	

All participants (applicant, applicant's representative, PTO personnel):

- (1) Michael V. Meller. (3) Carol Gruppi.
 (2) David Naff. (4) _____

Date of Interview: 18 December 2001 .

Type: a) ☒ Telephonic b) ☐ Video Conference
 c) ☐ Personal [copy given to: 1) ☐ applicant 2) ☐ applicant's representative]

Exhibit shown or demonstration conducted: d) ☐ Yes e) ☒ No.
 If Yes, brief description: _____ .

Claim(s) discussed: All pending .

Identification of prior art discussed: All of record .

Agreement with respect to the claims f) ☐ was reached. g) ☒ was not reached. h) ☐ N/A.

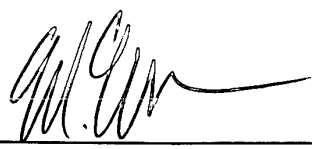
Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: Examiner discussed possible claim limitations of claim 1 to "sex-selected" and "flow cytometry". and limited to claims 10 and 11 .

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

i) ☐ It is not necessary for applicant to provide a separate record of the substance of the interview(if box is checked).

Unless the paragraph above has been checked, THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.



 Examiner's signature, if required

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record

A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.

All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:

- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case unless both applicant and examiner agree that the examiner will record same. Where the examiner agrees to record the substance of the interview, or when it is adequately recorded on the Form or in an attachment to the Form, the examiner should check the appropriate box at the bottom of the Form which informs the applicant that the submission of a separate record of the substance of the interview as a supplement to the Form is not required.

It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

- 1) A brief description of the nature of any exhibit shown or any demonstration conducted,
- 2) an identification of the claims discussed,
- 3) an identification of the specific prior art discussed,
- 4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
- 5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
- 6) a general indication of any other pertinent matters discussed, and
- 7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.



Docket No. 22091-701CON1

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a)):

[X] Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Commissioner for Patents, Washington D.C. 20231.

[] Pursuant to 37 C.F.R. § 1.6(d), I hereby certify that this paper and all enclosures are being sent via facsimile on the date indicated below to the attention of Examiner _____ at Facsimile No. _____ at _____ a.m./p.m.

Dated: March 7, 2002

Name of Person Certifying: Nancy L. Hug

Printed Name: Nancy L. Hug

#13
M.G.J.
3/22/02

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

RECEIVED
MAR 21 2002
TECH CENTER 1600/296

Applicant: John L. Schenk
Serial No.: 09/478,299
Filing Date: 01/05/2000
Title: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

Assignee: XY, Inc.
Examiner: M. Meller
Group Art Unit: 1651

Commissioner for Patents
Washington, D.C. 20231

PETITION AND FEE FOR EXTENSION OF TIME
(37 C.F.R. § 1.136(a))

Sir:

Pursuant to 37 C.F.R. § 1.136(a), Applicant hereby petitions for a 3 month extension of time to respond to the Office Action mailed on September 7, 2001.

1. The communication in connection with the matter for which this extension is requested

- a. ☒ is filed herewith; or
b. ☐ has been filed on _____.

2. ☒ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.

02/20/2002 AMANDA 00000153 501109 03470279
02 FCB217 450.00 CN

Docket No. _____

3. The following fees are submitted:

3. The following

	TOTAL MONTHS REQUESTED	OTHER THAN SMALL ENTITY	SMALL ENTITY	CALCULATIONS
a. ☐	one month	\$110.00	\$55.00	\$
b. ☐	two month	\$400.00	\$200.00	\$
c. ☒	three month	\$920.00	\$460.00	\$460.00
d. ☐	four month	\$1,440.00	\$720.00	\$
e. ☐	five month	\$1,960.00	\$980.00	\$
f. ☐	An extension for _ months has already been secured for filing the above-identified communication and the fee paid therefor of \$_____ is deducted from the total fee due for the total months of extension now requested. The fee for this extension (\$____), minus the fee previously paid (\$____) equals \$_____ (total fee due).			\$
TOTAL FEES =				\$460.00

- ☒ Conditional Petition for Extension of Time: An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$_____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701CON1, in the amount of \$460.00 to cover the above-fees. *A duplicate copy of this sheet is enclosed.*
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701CON1. *A duplicate copy of this sheet is enclosed.*

DATE: March 7, 2002

Respectfully submitted,

By: _____

Carol M. Gruppi

Registration No.: 37,341

Mailing Address

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center, Suite 1800
San Francisco, California 94111
Telephone: (650) 849-4400
Telefax: (650) 849-4800

MAR 18 2002

Docket No. 22091-0701CON1

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a)):

[x] Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Commissioner for Patents, Washington D.C. 20231.

[] Pursuant to 37 C.F.R. § 1.6(d), I hereby certify that this paper and all enclosures are being sent via facsimile on the date indicated below to the attention of Examiner _____ at Facsimile No. _____ at _____ a.m./p.m.

Dated: March 7, 2002

Name of Person Certifying: Nancy L Hug
Printed Name: Nancy L. Hug

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John L. Schenk
Serial No.: 09/478,299
Filing Date: 01/05/2000
Title: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

Assignee: XY, Inc.
Examiner: M. Meller
Group Art Unit: 1651

RECEIVED
MAR 21 2002
TECH CENTER 1600/2900

Commissioner for Patents
Washington, D.C. 20231

**NOTICE OF APPEAL TO THE BOARD OF PATENT APPEALS
AND INTERFERENCES**

Sir:

Applicant hereby appeals to the Board of Appeals from the decision dated September 7, 2001 of the Examiner rejecting claims 1-26 and 35. The items checked below are appropriate.

- ☒ Appeal Fee:
- ☐ Large Entity Fee of \$320.00; or
- ☒ Small Entity Fee of \$160.00.
- ☒ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.
- ☒ Petition for Extension of Time (3 mos.)
- ☒ Petition for Extension of Time Fee \$460.00
- ☒ Return Postcard.

03/20/2002 AWDNDF1 00000153 501189 09478299

01 FEB 2002 160.00 CM

52073644.1/22091-0701

☒ Payment of Fees

☐ Enclosed is Check No. _____ in the amount of \$ _____.

☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701CON1 in the amount of \$620.00 to cover the above-fees. *A duplicate copy of this sheet is enclosed.*

☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701CON1. *A duplicate copy of this sheet is enclosed.*

DATE: March 7, 2002

Respectfully submitted,

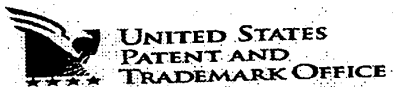
By: _____

Carol M. Gruppi

Registration No.: 37,341

Mailing Address

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center, Suite 1800
San Francisco, California 94111
Telephone: (650) 849-4400
Telefax: (650) 849-4800



Commissioner for Patents
Washington, DC 20231
www.uspto.gov

APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
09/478,299	01/05/2000	John L. Schenk	22091-701

CONFIRMATION NO. 1509



OC000000008851434

Craig Miles
Santangelo Law Offices P C
125 South Howes Street
3rd Floor
Fort Collins, CO 80521

Date Mailed: 09/25/2002

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 08/20/2002.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.


JAMES T WASHINGTON
LC106 (703) 308-9106

OFFICE COPY

10-09-02

GP 1651 #



Express Mail No: EV110549523US
Attorney Docket: 22091-701

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

In Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1651
Examiner: M. Meller
Assignee: XY, Inc.

RECEIVED
OCT 15 2002
TECH CENTER 1600/2900

LETTER OF TRANSMITTAL

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

Enclosed for filing are:

- 1) three copies of Appellants' Brief pursuant to 37 C.F.R. 1.192;
- 2) a Petition for Extension of Time;
- 3) a Request for Oral Hearing;
- 4) a check in the amount of \$1,280.00 payable to The Commissioner of Patents and Trademarks for an Appeal Brief (\$160.00), a Request for Oral Hearing (\$140.00), and a Petition for a five Month Extension of Time (\$980.00);
- 5) a Certificate of Express Mailing for each document;
- 6) this Letter of Transmittal and a postcard receipt for return to the undersigned.

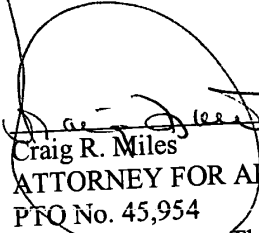
Please confirm receipt of the documents by applying your date stamp on the enclosed postcard receipt and returning it to me.

Dated this 7 day of October, 2002.

Respectfully Submitted,

SANTANGELO Law Offices, P.C.

By:


Craig R. Miles
ATTORNEY FOR APPLICANT
PTO No. 45,954
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

appealbr.xmt

#17/jlw
10.18.02



Express Mail No: EV110549523US
Attorney Docket: 22091-701

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

RECEIVED
OCT 15 2002
TECH CENTER 1600/2900

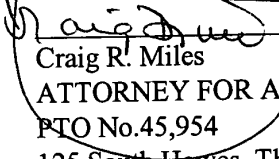
In Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1651
Examiner: M. Meller
Assignee: XY, Inc.

REQUEST FOR ORAL HEARING

Appellants request an oral hearing on the above appeal. The fee required by 35 U.S.C. §41(a)(6) and 37 C.F.R. §1.17(g) for this request is enclosed.

Dated this 7 day of October, 2002.

Respectfully Submitted,
SANTANGELO Law Offices, P.C.

By: 
Craig R. Miles
ATTORNEY FOR APPLICANT
PTO No. 45,954
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

10/11/2002 DTESSEH1 00000064 09478299
02 FC:221 140.00 OP



Express Mail No: EV110549523US
Attorney Docket: 22091-701

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

In Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1651
Examiner: M. Meller
Assignee: XY, Inc.

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CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day deposited the attached Request for Oral Hearing with the United States Postal Service as Express Mail, postage prepaid, in an envelope addressed to: The Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Dated this 7 day of October, 2002.

Cheryl A. Swanson
Cheryl A. Swanson



#16/10
10-18-02
Express Mail No: EV110549523US
Attorney Docket: 22091-701

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

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PETITION FOR EXTENSION OF TIME UNDER 37 C.F.R. § 1.136(a)

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

On behalf of the applicant, the undersigned attorney hereby requests an extension of time pursuant to 37 C.F.R. § 1.136(a) and 37 C.F.R. § 1.17(a)(5). This request is submitted in relation to a Notice of Appeal mailed March 7, 2002 for application number 90/478,299, having two months to file an Appeal Brief. The Appeal Brief would otherwise be due May 7, 2002; however, it is requested that this time period be extended to and including October 7, 2002. This extension will thereby encompass an additional five months of the extension of time available pursuant to 37 C.F.R. § 1.136(a). Pursuant to 37 C.F.R. § 1.17(a)(5), an extension fee of \$980.00 is included with this Petition and the accompanying Appeal Brief. In the event the fee has been

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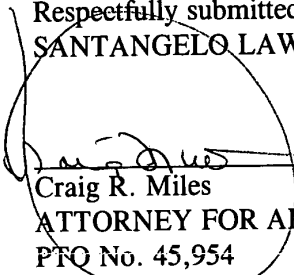
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inappropriately calculated, please allow the maximum extension possible and inform me at your earliest convenience so that any deficiency or error can be corrected immediately.

Dated this 7 day of October, 2002.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By:


Craig R. Miles
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Express Mail No: EV110549523US
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APPELLANT'S BRIEF PURSUANT TO 37 C.F.R. §1.192

Honorable Commissioner
of Patents and Trademarks
Washington, D.C. 20231

REAL PARTY IN INTEREST

The subject application is owned by XY, Inc. whose current address is Moondrift
Ranch, 1108 North Lemay Avenue, Fort Collins, Colorado 80524, U.S.A., a Colorado
Corporation.

RELATED APPEALS AND INTERFERENCES

None.

U.S. PATENT & TRADEMARK OFFICE

NO. 1000000

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STATUS OF CLAIMS

On March 7, 2002, appellant appealed from the final rejections of claims 1-26 and 35. Claims 27-34 and claims 36 and 37 were cancelled without prejudice in appellant's response to the official communication mailed April 12, 2000 pursuant to a restriction requirement.

STATUS OF AMENDMENTS

Claims 1-26, and 35 have not been amended during examination of the application. Hence, claims 1-26 and 35 are in the form as originally filed.

SUMMARY OF THE INVENTION

Appellant's invention comprises a method of cryopreservation of sperm cells that have been selected for a particular characteristic and the corresponding frozen sperm cells selected for a particular characteristic that are fertile and can be successfully used for artificial insemination in vivo to achieve pregnancies or for in vitro fertilization to produce embryos.

The examiner imposed a restriction requirement that included the method of cryopreservation of sperm cells that have been selected for a particular characteristic and a product by that method in a first group (Group I, claims 1-26 and 35); the frozen sperm

selected for a particular characteristic in a second group (Group II, claims 27-34); and the method of using the product in a third group (Group III, claims 36 and 37). The examiner further imposed an election of species requirement that necessitated election of one of the patentably distinct species set out by claim 12 and requiring the applicant to identify one specific cryoprotectant and one specific additional component. Group I was elected, and that is the group on appeal. The elected claims in Group I, are set forth in Appendix B to this brief.

Appellant's method of freezing sperm cells that have been selected for a particular characteristic (claims 1-26) and a product by such method (claim 35) (comprising elected Group I), provides selected frozen sperm cells that when thawed can be used to successfully inseminate a female mammals and achieve pregnancies or used in vitro for fertilization of oocytes. Specification at page 3, lines 9-12. Selected sperm cells comprise sperm cells isolated from the normal population of sperm cells in ejaculates of male mammals based upon the presence or absence of a specific characteristic. Specification at page 3, lines 9-10 and page 4, lines 9-10. Thus, a selected sperm sample is obtained by subjecting the normal sperm cell population in an ejaculate of a male mammal to selection for a specific characteristic. Specification at p. 4, lines 11-12. A selected sperm sample is therefore enriched in the characteristic selected for, relative to the normal sperm cell population in an ejaculate of a male mammal. Specification at p. 4, lines 12-13. Selected sperm samples are then frozen. Specification at page 3, lines 9-10. Thawing of frozen selected sperm cells yields viable sperm that can be used in procedures such as artificial insemination and in vitro fertilization. Specification at page 3, lines 11-12.

The various embodiments of the invention can further include frozen selected sperm cells that have a concentration of frozen selected sperm cells that is lower than the concentration of normal populations of sperm cells in ejaculates. Specification at page 15, lines 24-28, Claim 2. The concentration of frozen selected sperm cells with respect to some embodiments of the invention can be about $1 \times 10^6/\text{mL}$ to about $300 \times 10^6/\text{mL}$. Specification at page 11, lines 25-26.

The various embodiments of the invention can further include, sex-selecting frozen sperm cells to establish either enriched X-chromosome bearing or Y-chromosome bearing populations as compared to normal sperm cell populations of ejaculates. Specification at page 5, lines 25-30, page 6, lines 1-6; Claim 3. The sperm cells contained in ejaculates of various mammals, including, but not limited to, bovine sperm cells, equine sperm cells, porcine sperm cells, ovine sperm cells, elk sperm cells, or bison sperm cells can be sex-selected. Specification at page 5, lines 13-17; Claims 4-7. Various procedures can be used to sex-select sperm cells obtained from such male mammals including but not limited to magnetic techniques, gravimetric techniques, biochemical techniques, sperm cell motility techniques among others described. Specification at page 5, lines 25-31 and page 6, lines 6; Claim 8. The invention further specifically includes freezing of sex-selected cells obtained by flow sorting of sperm cells contained in ejaculates by flow cytometry. Specification at page 6, lines 7-19; Claim 9.

The various embodiments of the invention further include cooling of frozen selected sperm cells at 5° degrees Celsius prior to freezing. Specification at page 10, lines 26-28; Claim 10. Suitable conditions for cooling of selected sperm cells can comprise an extended cooling period of between about 60 minutes to about 240 minutes. Specification at page 10, lines 29-31, page 11, lines 1-3; Claim 11.

Embodiments of the invention can further comprise adding a final extender to selected sperm cells that contains a cryoprotectant compatible with selected sperm cell to be frozen, such as, glycerol, dimethyl sulfoxide, ethylene glycol, or propylene glycol. Specification at page 8, lines 4-14; Claim 14.

Embodiments of the invention can further comprise adding a final extender to selected sperm samples that contains, in addition to a cryoprotectant, one or more of the following components: a component that maintains osmolality and buffers pH, an organic substance that reduces cold shock and preserves fertility of sperm, an energy source, a substance that facilitates sperm capacitation, and an antibiotic. Claim 12. Osmolality and pH can be maintained by establishing a buffer containing a salt, a carbohydrate, or specifically Tris[hydroxymethyl]aminomethane, and TES (N-Tris [hydroxymethyl]methyl-2-aminomethanesulfonic acid), and monosodium glutamate buffers; milk; or HEPES-buffered medium. Specification at page 8, lines 26; Claims 15 and 16. Cold shock can be reduced and fertility of sperm preserved using organic substances such as lipoproteins, phospholipids, lecithin, egg yolk, egg yolk extract, milk, milk extract, casein, albumin, or a detergent such as sodium dodecyl sulfate. Specification at page 9, lines 5-7; Claim 17.

Energy sources that can be readily utilized by selected sperm cells can include for example sugars such as monossaccharides, disaccharides, trisaccharides, glucose, fructose, or mannose. Specification at page 9, lines 17-23; Claims 13, 14, and 18. Substances that facilitate sperm capacitation can be enzymes such as alpha amylase, beta amylase, beta glucuronidase. Specification at page 9, lines 29-30, page 10, lines 1-2. Antibiotics such as tylosin, gentamicin, lincomycin, spectinomycin, linco-spectin, penicillin, streptomycin, ticarcillin although any variety of antibiotics or combinations thereof compatible with the species of selected sperm cell can be used. Specification at page 10, lines 6-10.

As to some embodiments of the invention, isolating selected sperm cells from the selected sperm sample in initial extender can be accomplished by centrifugation. Specification at p. 11, lines 6 and 13; Claim 24. Centrifugation in accordance with the invention can isolate at least 50% to about 90% of the selected sperm cells in the selected sperm sample. Specification page 11, line 17; Claim 25.

Certain embodiments of the invention can further provide frozen selected sperm cells in a final extender has a pH range of about 6.5 to about 7.5. Claim 23

Certain embodiments of the invention provide a suspension of selected sperm cells in a mixture prior to freezing of glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

Specification at page 12, lines 3-7; page 13, table entitled "Components of Egg Yolk-Tris Extender"; Claim 20.

Other embodiments of the invention provide a suspension of selected sperm cells in a mixture prior to freezing of glycerol, sodium citrate, egg yolk, and one or more antibiotics. Specification at page 13, lines 18-22; page 14, table entitled "Components of Egg-Citrate Extender"; Claim 21.

Another embodiment of the invention provides a suspension of selected sperm cells in a mixture prior to freezing of glycerol, heated homogenized milk, fructose, and one or more antibiotics. Specification at page 15, lines 17-21; page 16, table entitled Components of Milk Extender; Claim 22.

Importantly, the inventors have demonstrated, for the first time, that frozen selected sperm cell products resulting from the various embodiments of the invention can be thawed and used to inseminate female mammals and successfully achieve pregnancies or used for in vitro for fertilization of oocytes. Specification at page 3, lines 9-12. The ability to freeze selected sperm cells, which the invention provides, will enable widespread use of selected sperm cells. Specification page 3, lines 22-24. As such, the invention represents an important advance in livestock management, where selection of sperm cells for use in such procedures can be used to increase the production of offspring having desirable traits. Specification at page 3, lines 17-19.

ISSUES

1. Whether the examiner incorrectly rejected the claims 1 and 35 under Section 112, second paragraph, as being indefinite and failing to particularly point out and distinctly define the metes and bounds of the invention?
2. Whether the examiner incorrectly rejected claims 1-26 and 35 under 35 U.S.C. §103(a) as unpatentable over Salisbury et al, Physiology of Reproduction and Artificial Insemination of Cattle, 2nd Ed., San Francisco: W. H. Freeman, 442-554 (1978) in view of United States Patent No. 5,021,244, issued on June 4, 1991, to Spaulding.

GROUPING OF CLAIMS

Claims 1-26 and 35 stand or fall together.

EXAMINER'S RATIONALE

The examiner's rationale for rejecting claims 1-26 and 35 as unpatentable over Salisbury et al, Physiology of Reproduction and Artificial Insemination of Cattle, 2nd Ed., San Francisco: W. H. Freeman, 442-554 (1978) in view of United States Patent No. 5,021,244, issued on June 4, 1991, to Spaulding was stated in his first office action as follows:

“Claims 1-26 and 35 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

In claims 1 and 35, step c) which calls for “isolating sperm from said selected sperm sample to produce isolated sperm”, is confusing since a sperm sample is isolated

once it is no longer in the testicles. Step c, is thus redundant in its recitation of "isolating sperm" since the sperm is already isolated.

Further, the claim is confusing since the term, "final extender" is confusing. What is the final extender final to? There is no beginning extender." and;

"Claims 1-26 and 35 are rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. taken with Spaulding.

The claims are drawn to a method for the cryopreservation of sperm as in claims 1 and 35 using an extender the elected extender containing glycerol and a component that maintains osmolality and buffers pH.

Salisbury teaches cryopreservation of bull semen using different extenders including glycerol (page 487) and a component that maintains osmolality and buffers pH as sodium citrate, Tris, milk, etc. Salisbury also teaches obtaining the sperm cooling it to 5 C for 140 minutes (see page 475-see also pages 463-464 for additional cooling semen), adding the extender (see page 456, 499), and freezing the suspension of sperm (see pages 471, 494, 495, 503-504). The pH of the extender is 6.5 to 7.0 (page 502 and page 456). See entire reference especially pages mentioned.

Salisbury does not teach to specifically isolate the sperm using centrifugation and does not teach using flow cytometry to select the sperm sample.

Spaulding teaches to sort sperm for their X or Y characteristic, by flow cytometry and to centrifuge sperm cells to remove seminal proteins, i.e. isolate the sperm, after cooling, see example 1.

It would have been obvious to one of ordinary skill in the art at the time the invention was made to use flow cytometry to select sperm for a particular characteristic in the method of Salisbury since Spaulding teaches using flow cytometry to sort X from Y sperm. To centrifuge (isolate) the sperm sample of Salisbury after cooling would have been obvious since Spaulding teaches to remove seminal plasma proteins, i.e. isolate the sperm after cooling.

Since Salisbury uses bull sperm it also would have been obvious to use other mammalian sperm such as equine and porcine since they are also mammalian sperm and would be expected to work well within the purview of the skilled artisan.

A 50% to 90% recovery of sperm from the centrifugation is obvious since one would want to yield as much of the sperm possible.

The concentration of sperm in said suspension prior to freezing would be inherent to the suspension of sperm.

The order of the steps of the process of Salisbury might be out of order from the claimed process, but this is still obvious. MPEP 2144.04 IV (C) states that it is prima facie obvious to perform in any order steps of a process.

Thus, the claimed subject matter is obvious over the cited references.”

The examiner's rational for rejecting claims 1-26 and as unpatentable over Salisbury et al, Physiology of Reproduction and Artificial Insemination of Cattle, 2nd Ed., San Francisco: W. H. Freeman, 442-554 (1978) in view of United States Patent No. 5,021,244, issued on June 4, 1991, to Spaulding was further set forth in the final rejection as follows:

“Applicants argue that Salisbury does not teach or suggest that sperm selected for a particular characteristic may be frozen or that pregnancies can be achieved with such selected cryopreserved sperm. In response to applicants arguments against the references individually, one cannot show nonobviousness by attacking the references individually where the rejections are based on a combination of references. See *In Re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In Re Merck & Co.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986).

Applicant cannot expect Salisbury to contain all the elements of the claims since Salisbury was used in a 35 USC 103 rejection taken with Spaulding.

Further, Salisbury does teach freezing of sperm and Spaulding teaches the selection of the sperm for a particular characteristic, namely, sex.

Also, the elected claims are not directed to pregnancies resulting from the use of the sperm, thus this argument by the applicant is moot.

Thus, the claimed subject matter is properly rejected under this section.”

ARGUMENT

I. Claims 1 and 35 Should Not Have Been Rejected Under Section 112 Second Paragraph As They Are Sufficiently Definite And Particularly Point Out And Distinctly Define The Metes And Bounds Of The Invention.

The applicant believes that the claims as recited are both definite and particular point out and distinctly define the metes and bounds of the invent as required under 35 U.S.C. §112, second paragraph.

Section 112, second paragraph sets out two separate requirements:

“(A) the claims must set forth the subject matter that applicants regard as their invention; and

(B) the claims must particularly poihtout and distinctly define the metes and bounds of the subject matter that will be protected by the patent grant.”

In satisfying the requirements of §112, the meaning of words used in a claim are not to be construed in a "lexicographic vacuum, but in the context of the specification and drawings. MPEP §2106(C). "An applicant is entitled to be his or her own lexicographer, and in many instances will provide an explicit definition for certain terms used in the claims. Where an explicit definition is provided by the applicant for a term, that definition will control interpretation as it is used in the claim." MPEP §2106(C); Toro Col v White Consolidated, 199 F.3d 1295, 1301, 53 USPQ 1065, 1069 (Fed. Cir. 1999) (emphasis added). Office personnel must rely on the applicant's disclosure to properly determine the meaning of the terms used in the claims. MPEP §2106 (C); Markman v. West view Instruments, 52 F.3d 967, 980, 34 USPQ2d 1321, 1330 (Fed. Cir.)(en banc), affd 116 S.Ct. 1384 (1996).

If a rejection is based on §112, second paragraph, the examiner should further explain whether the rejection is based on indefiniteness or on the failure to claim what applicants regard as their invention. MPEP §2171; Ex parte Ionescu, 222 USPQ 537, 539 (Bd. App. 1984).

In the instant case the examiner indicates that the claims are rejected under §112 "as being indefinite for failing to particularly point out and distinctly claim the subject matter of which the applicant regards as the invention." Office Action Summary, page 3, item 3. It appears to the appellant that the examiner has combined concerns regarding "indefiniteness" and the "failure to claim what applicants regard as there invention" to the extent that the applicant could not reasonably understand which concern to address. *Id.* As a matter of convenience for the Board the applicant will address both as follows:

The examiner expressed the concern that in "claim 1 and 35, step c) which calls for 'isolating sperm from said selected sperm sample to produce isolated sperm is confusing." Office Action Summary mailed December 19, 2000, page 3, item 3.

In the specification under the heading entitled "Isolation of Sperm Cells from the Selected Sperm Sample" the appellant provides an explicit definition of the term "isolation" as meaning "after initial extension of the selected sperm, sperm are isolated from the sample using any sufficiently gentle isolation method that provides at least 50% recovery of sperm. ...". Specification at page 11, lines 5-8. The applicant further provides

an explicit definition of the term "initial extender" as "a medium used to extend sperm prior to isolation step of the method of this invention". Specification at page 4, lines 18-19. The applicant further provides detailed examples of "a variety of methods suitable for recovering cells from suspension to isolate sperm. Specification page 11, starting at line 12. These examples include for example, "filtration, sedimentation, and centrifugation. . . In an exemplary preferred embodiment, the selected sperm is aliquoted into 50 ml tubes at volumes not exceeding 27ml. . .the centrifugation step provides at least about 50%. . .recovery of sperm. . .After isolation, the supernatant is removed and the pellet is suspended by vortexing gently. . . Specification at page 11, lines 13-20.

Because appellant has acted as his own lexicographer and explicitly defined the term "isolating sperm" in the specification in a manner which would be apparent to any person of ordinary skill in the art at the time the invention was made as to what the appellant regards as his invention, and also particularly points and distinctly defines the metes and bounds of the subject matter to be protected by the patent grant, and because appellant's definition is not repugnant to the usual meaning of the term, appellants definition of the term should be allowed to control the interpretation of the claims and should not have been rejected by the examiner. Certainly, no person of ordinary skill in the art would interpret "isolating sperm" as the examiner suggests "a sperm sample is isolated once it is no longer in the testicles."

The examiner also rejected under §112, second paragraph, the term "final extender" as "confusing". Office Action Summary mailed December 19, 2000, page 3,

item 3. In the Specification, under the heading "Definitions" the appellant has explicitly defined both "initial extender" as meaning "a medium used to extend sperm prior to the isolation step of the method of this invention" and "final extender" as "a medium used to extend sperm prior to the freezing step of the method of the invention". Specification page 4, lines 18-21. Additionally, in the specification under the heading "Final Extension of Isolated Sperm Cells" the appellant clearly indicates that "After isolation, the sperm are pooled. . .and extended with final extender to an appropriate concentration for freezing." Specification at page 11, lines 23-25. The appellant then provides various types of suitable final extenders as examples. Specification at page 11, line 29 through page 16, line 21.

Again, because appellant has acted as his own lexicographer and explicitly defined the terms "initial extender" and "final extender" in the specification in a manner which would be apparent to any person of ordinary skill in the art at the time the invention was made as to what the appellant regards as his invention, and also particularly points and distinctly defines the metes and bounds of the subject matter to be protected by the patent grant, and because appellant's definition is not repugnant to the usual meaning of the term, appellants definition of the term should be allowed to control the interpretation of the claims and should not have been rejected by the examiner.

Moreover, the appellant selected both terms "isolating sperm" and "final extender" as generic terms to include the wide variation in the manner of isolating sperm,

and to further include the numerous compositions of final extenders described in the specification and which can be used with the invention.

The appellant respectfully requests that the Board reverse the examiner's rejection allowing the appellant to retain the terms "isolating sperm" and "final extender" as originally recited in the specification as filed because they meet both standards set by §112, second paragraph, and provide appellant with the breadth of claim scope that fairly represents his invention.

II. The Examiner Has Not Established A Case of Obviousness Under Section 103 By Rejection Of Claims 1-26 and 35 As Unpatentable Over Salisbury et al. In View Of Spaulding.

The appellant believes that the examiner has not established a prima facie case of obviousness with respect to the appellant's claimed method of cryopreserving sperm using the combination of Salisbury et al, Physiology of Reproduction and Artificial Insemination of Cattle, 2nd Ed., San Francisco: W. H. Freeman, 442-554 (1978) (Salisbury) in view of United States Patent No. 5,021,244, issued on June 4, 1991, to Spaulding (Spaulding).

A prima facie case of obviousness requires that the references in combination teach or suggest all the claim limitations; there must be some suggestion or motivation, either in the reference themselves or in the knowledge generally available to one of ordinary skill of the art to modify the reference or combine reference teachings; and there

must be a reasonable expectation of success. §2143, MPEP; In re Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

First, the combination of references does not teach or suggest all the limitations of the claimed invention as required. §2143, MPEP; §2143.03, MPEP; In re Royka, 490 F.2d 981 (CCPA 1974).

In this regard, with respect to claims 1 and 35, the examiner has failed to use the appellant's explicit definition of the term "sperm" set out in the specification, as required under the rules. As discussed at length by appellant above, "office personnel must rely on the applicant's disclosure to properly determine the meaning of the terms used in the claims. MPEP §2106 (C); Markman v. West view Instruments, 52 F.3d 967, 980, 34 USPQ2d 1321, 1330 (Fed. Cir.)(en banc), *aff'd* 116 S.Ct. 1384 (1996). "An applicant is entitled to be his or her own lexicographer, and in many instances will provide an explicit definition for certain terms used in the claims. Where an explicit definition is provided by the applicant for a term, that definition will control interpretation as it is used in the claim." MPEP, §2106(C); Toro Co. v. White Consolidated, 199 F.3d 1295, 1301, 53 USPQ 1065, 1069 (Fed. Cir. 1999) (emphasis added).

The applicant has explicitly defined the term "sperm" as used in claim one to mean "fertile sperm". Specification at page 3, lines 11-12; Appellants Response Under 37 C.F.R. §1.111 at page 2, ¶4. As can be explicitly understood from the specification and appellant's response to the office's first action, "The present inventors have

demonstrated, for the first time, that pregnancies can be achieved with sperm that has been selected and then frozen." Specification at page 3, lines 15-16 (emphasis added). "The present invention allows cryopreservation of sperm that have been selected for a particular characteristic. . .Thawing yields viable sperm that can be used in procedures such as artificial insemination and in vitro fertilization." Specification at page 3, lines 9-12.

The examiner, in order to make the combination of references teach each element of claim 1, impermissibly broadens appellant's explicit definition of "selected sperm sample" to include the products taught by Spaulding regardless of the fact that there is no evidence within the combination of references that these products include any "viable sperm" which could be used to fertilize a female mammal to achieve pregnancies.

The Salisbury reference cited by the examiner makes it unequivocally clear why the appellant's explicit definition of "selected sperm sample" and evidence of pregnancies is necessary in understanding the meaning and scope of claim 1:

"Spermatozoa must be alive to be fertile. . .and motility has been used to monitor viability. Motility, however, is primarily a reflection of flagellar activity, and it does not guarantee that such cells are fertile. The integrity of the genetic material in the sperm chromatin must also be preserved in order for normal embryo development to take place after fertilization. . .there is no test of semen quality prior to use that can predict how well the genetic material is maintained."

Salisbury at page 442.

When the explicit definition set out by the appellant in the specification and claim 1 is properly interpreted it can be understood that the combination of references do not at teach the claimed limitation of "selected sperm cells" of the invention because Salisbury does not teach "selected sperm cells" at all and not a single pregnancy can be attributed to the technology described by Spaulding. This is not surprising in that although the combined references are 132 pages in length there is only a two sentence description of any application of freezing to any type of selected sperm cells and there is no evidence any of the selected sperm cells are fertile selected sperm cells. Spaulding, col. 18, lines 30-34. As can be understood, the Spaulding reference carefully avoids any indication what-so-ever that the mixture of sperm cells modified with X- or Y- SAM proteins can actually fertilize an egg or that any pregnancy can be achieved. As such, there is no indication that the appellant's definition of "sperm" which is limited to frozen selected fertile sperm cells includes any of the sperm cells described in the combination of references.

Because the none of the references in the combination of Salisbury et al. in view of Spaulding disclose, teach, or suggest the claimed limitation of "selected sperm cells" as explicitly defined in the specification as being fertile, a prima facie case of obviousness cannot be established with regard to the claim limitations in claim 1, nor any claim depending there from. § 2143.03, MPEP; In re Fine, 837 F.2d 1071 (Fed. Cir. 1988).

As such, respectfully request that the examiners rejection of claims 1-27 and 35 be reversed and claims 1-26 and 35 should have been allowed as originally recited, or amended to further clarify fertile sperm cells.

Secondly, the references do not provide the requisite suggestion or motivation to combine the references as required by § 2143.01, MPEP. The mere fact that references can be combined does not render the resultant combination obvious unless the prior art also suggests the desirability of the claimed invention. §2143.01, MPEP; In Re Mills, 916 F.2d 680 (Fed. Cir. 1990).

As the examiner indicates the "Salisbury reference it teaches cryopreservation of bull semen using different extenders." Office Action Summary mailed December 19, 2000 at page 4, item 5 (emphasis added). The examiner points out that "Salisbury does not teach to specifically isolate the sperm using centrifugation and does not teach flow cytometry to select the sperm sample." Office Action Summary mailed December 19, 2000 at page 4, item 5.

Importantly, the examiner omits to point out that Salisbury the primary reference does not teach the first element of appellants invention "obtaining a selected sperm sample". Claim 1, element a. This is an important difference because "selected sperm samples" are "obtained by subjecting a source sample to selection based on a specific

characteristic." Specification at page 4, lines 9-13. As such, the Salisbury reference does not teach any of the elements of the claimed invention:

- a. obtaining a selected sperm sample;
- b. cooling said selected sperm sample;
- c. isolating sperm from said selected sperm sample;
- d. adding final extender to said isolated sperm to produce a suspension of sperm; and
- e. freezing said suspension of sperm."

Claim 1, Specification at page 55. (emphasis underline added to highlight that "selected sperm" not semen or sperm cells contained within semen are the subject matter of the claimed invention).

There is no relationship what-so-ever between semen, or sperm cells contained within semen, as taught by Salisbury to the "selected sperm cells" which are used in accordance with the invention. First, it is apparent from the specification that "selected sperm cells" are not a natural product; selected sperm cells are removed from the seminal plasma; selected by a characteristic to establish a non-naturally occurring population of sperm cells purified for a particular characteristic (specification at page 4, ll. 9-15, including but not limited to sex-selected sperm, specification at page 17, ll. 19-25); can contain a variety of components besides selected sperm cells (specification page 7, ll. 7); and may as a consequence of the selection process be modified to the extent that they are not sperm cells in the conventional sense, at all (specification at page 5, lines 9-15; page 17, lines 19-25; page 6, lines 7-19) (describing stain bound to DNA of selected sperm

cells obtained by flow cytometry). As such, the Salisbury reference does not disclose any limitation of the invention (and arguably is not even a pertinent reference given that the reference itself indicates that it is "impossible to generalize findings" as "one set of conditions may not be acceptable for others"). Salisbury at page 499. As such, within the combination of references the examiner must rely upon the Spaulding reference to show that limitations of the invention were taught, and to provide a suggestion that it can be combined at all with the Salisbury reference.

The Spaulding reference focuses on producing a "polyclonal or monoclonal antibodies. . . useful to modify mammalian semen." Spaulding, col. 17, ll. 61-62. "When incubated with a semen sample. . .Sperm bound with antibodies become inactivated--their mobility is impeded." Spaulding, col. 17, ll. 67-68, col. 18, l. 1 (emphasis added to highlight that Spaulding obtains semen not selected sperm cells). "The semen was either collected and frozen in liquid nitrogen, or it may be freshly ejaculated." Spaulding, col. 18, ll. 15-16. "antibodies are incubated with the semen sample for 15-60 min at 37 C. Following incubation, the mixture is used directly for artificial insemination (AI) or is frozen in liquid nitrogen according to standard procedures." Spaulding, col. 18, ll. 30-34. As can be understood, in the entire Spaulding reference the indication that " or is frozen in liquid nitrogen according to standard procedures" is the only reference to any freezing step. Spaulding col. 18, 33-34. However, in combination with Salisbury this singular statement does not suggest the desirability of the claimed invention.

The mere fact that the references can be combined or that the combination could be modified does not render the invention obvious unless the references also suggest the desirability of the invention. MPEP, §2143.01; In re Mills, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990)("Although a prior art device may be capable of being modified. . .there must be a suggestion or motivation in the reference to do so." Id at 682, 1432). The combination of Salisbury and Spaulding do not teach freezing of "selected sperm cells" removed from the seminal plasma. Moreover, Spaulding does not suggest in any manner that freezing of semen as taught by Salisbury, or any conventional freezing technology for that matter, should be modified to operate with "selected sperm cells" which are removed from the seminal plasma.

The examiner further argues that because "Spaulding teaches to sort sperm for their X or Y characteristics, by flow cytometry and to centrifuge sperm cells to remove seminal proteins, i.e. isolate the sperm, after cooling . . .It would have been obvious to one of ordinary skill in the art at the time the invention was made to use flow cytometry to select sperm for a particular characteristic in the method of Salisbury since Spaulding teaches flow cytometry to sort X from Y sperm. To centrifuge (isolate) the sperm sample of Salisbury after cooling would have been obvious since Spaulding teaches to remove seminal plasma proteins, i.e. isolate the sperm after cooling. Office Action Summary mailed December 19, 2000 at page 5, item 5.

However, the examiners argument amounts to nothing more than a statement that flow sorting of stained sperm cells and centrifugation of flow sorted sperm cells was

known in the art, bald of any objective reason to combine the teaching with the prior art. The fact that the claimed invention is within the capabilities of one of ordinary skill in the art is not sufficient by itself to establish a prima facie case of obviousness. MPEP, §2143.01; Ex parte Levengood, 28 USPQ2d 1300 (Bd. Pat. App. & Interfer. 1993).

Spaulding does not teach, nor does it suggest that, sorted sperm cells (or any selected sperm cells which are isolated), can be frozen for any purpose. Spaulding collected subpopulations of sperm cells by flow cytometry "for isolation and identification of the novel, enriched plasma membrane vesicles and the SAM proteins. . . ." Spaulding, col. 10, ll. 3-5. "We cavitated cell samples containing enriched X-sperm or Y-sperm populations in Parr bombs." Spaulding, col. 10, ll. 18-19.

With respect to claims 5-7, having limitations which are not disclosed by either reference as discussed above, the examiner attempts to dispose of these limitations in a similar fashion by merely indicating that "equine and porcine since they are also mammalian sperm . . . would be expected to work well with the purview of the skilled artisan." . Office Action Summary mailed December 19, 2000 at page 5, item 5. Again, this argument is nothing more an argument that it was well within the skill of the art which is not sufficient by itself to establish a prima facie case of obviousness. MPEP, §2143.01; Ex parte Levengood, 28 USPQ2d 1300 (Bd. Pat. App. & Interfer. 1993).

With respect, to claim 25, the examiner again uses the impermissible "well within the ordinary skill of the art" argument that "a 50% to 90% recovery of sperm from the centrifugation is obvious since one would want to yield as much sperm as possible.

With respect to claims 2 and 26, the examiner urges that the concentration of the suspension of cells claimed would be "inherent" to the suspension itself. However, freezing this concentration of "selected sperm cells" is not taught by either reference and there is no suggestion in the Spaulding reference to apply the teaching of Salisbury to that concentration of selected sperm cells. To the contrary, the teaching of Salisbury conflicts with any attempt to combine the references and would render the Salisbury reference inoperable. "In New Zealand, . . . when frozen semen has been banked. . . the semen is thawed and reconstituted. . . with 20 million sperm per dose." Salisbury p. 475.

With respect to claim 23, the pH of the extender claimed by appellant for selected sperm cells is not used either by Spaulding or Salisbury for freezing selected sperm cells and Spaulding does not suggest that the teachings of Salisbury should apply to isolated selected sperm cells.

Because the references in the combination of Salisbury et al. in view of Spaulding do not provide the requisite motivation or suggestion for combination a prima facie case of obviousness cannot be established with regard to the claim limitations in claim 1, nor any claim depending there from. § 2143.03, MPEP; In re Fine, 837 F.2d 1071 (Fed. Cir. 1988).

As such, respectfully request that the examiners rejection of claims 1-27 and 35 be reversed and claims 1-26 and 35 be allowed as originally recited, or amended to further clarify fertile sperm cells.

Fourth, the combination of references does not provide one of ordinary skill in the art a reasonable expectation of successfully making the invention at the time the invention was made. §2143.02, MPEP. Where the references provide only a general approach as to the particular form of the claimed invention or how to achieve it, the invention is not obvious, but only obvious -to-try. In Re O'Farrell, 853 F. 2d 894, 903 (Fed. Cir. 1988).

With respect to claims 1-26 and 35, the teaching described by Salisbury does not provide any description of how to make, isolate, or freeze the "selected sperm cells" claimed by the appellant in claims 1-26 and 35. It only provides teachings as to how to freeze semen which comes with a variety of disclaimers and warnings that "the exact nature of freeze thaw damage is not fully understood" (page 497); that "factors in seminal processing make it impossible to generalize these findings (page 499); there is no test for semen quality prior to use that can predict how well the genetic material is maintained (page 442) and so forth. The Spaulding reference does not provide any description of how to freeze isolated selected sperm cells because the few sperm cells that represented isolated selected subpopulations were destroyed by cavitation and not frozen at all. Spaulding, col. 10, ll. 18-19. The only description in Salisbury pertains to sperm cells in

seminal plasma and to freeze these sperm cells "according to standard procedures". Spaulding, col. 18, ll. 33-34. This provides nothing more than is offered by Salisbury (which are standard procedures) which does not address any aspect of freezing with regard to selected sperm cells as claimed by appellant and comes with all the admonitions of Spaulding that it may not work as to any given set of circumstances.

Specifically with respect to claims 2 and 26, there is no description at all of freezing selected sperm cells at a concentration lower than in the source sample or in the range of 1×10^6 /ml to about 300×10^6 /ml. This range being important to the appellant because selected sperm cells are scarce; while it was unimportant when nearly unlimited sperm cells are available when freezing semen. Moreover, again the Salisbury reference indicates that "dilution of the spermatozoa with simple solutions depresses motility". Salisbury at page 446; See also specification at page 22, ll. 30-31 (high sperm dilution and cooling resulted in a substantial reduction in the percentage of motile sperm. . .this was greatly attenuated by concentrating the diluted sperm to 10×10^6 /ml). As such, it is surprising that any pregnancies were achieved.

Specifically with respect to claims 3-7, there is no description in the combination of references with respect to any sperm cells obtained from any source other than bull. As such, the combination of references does not provide any details at all as to how to make or use frozen selected equine sperm cells or frozen selected porcine sperm cells as claimed.

With respect to claim 8, the combination of references provides no description with regard to freezing sperm selected by flow cytometry at all. Limiting any description to killing flow sorted sperm by cavitation to harvest the resultant membranes and proteins.

Because the references in the combination of Salisbury et al. in view of Spaulding do not provide the requisite expectation of success at the time the invention was made a prima facie case of obviousness cannot be established with regard to the claim limitations in claim 1, nor any claim depending there from. § 2143.03, MPEP; In re Fine, 837 F.2d 1071 (Fed. Cir. 1988).

As such, appellant respectfully requests that the examiners rejection of claims 1-27 and 35 be reversed and claims 1-26 and 35 be allowed as originally recited, or as amended to further clarify fertile sperm cells.

In addition to the arguments set forth above concerning the failure to make the prima facie case of obviousness, the invention also provides unexpected advantages as compared to the combination of references cited by the examiner. Evidence of unexpected advantageous properties can rebut a prima facie case of obviousness. §716.02(a), MPEP; In Re Chupp, 816 F.2d 643, 646 (Fed. Cir. 1986).

As indicated by the Salisbury reference the only test that can predict how well the genetic material of sperm cells is maintained by a process is use. Salisbury at page 442.

The "ultimate test of a procedure is fertility. . . ." Salisbury at page 534. This is true because "the exact nature of total freeze-thaw damage is not fully understood." Salisbury at page 497. Reports of superior survival and fertility in other extenders are numerous, but possible interactions with other factors in seminal processing make it impossible to generalize these findings; a satisfactory extender for one animal or one set of conditions may not be acceptable for others. Salisbury at page 499.

As can be understood from scrutinizing the combination of references, there is no showing of any use of any selected frozen sperm cells sufficient under the test of Salisbury to prove fertility of any type of selected frozen sperm cells. No frozen selected sperm cell has been shown by the combination of references to actually achieve fertilization of even a single egg of any kind female mammal, whether in vitro or in vivo.

Even with respect to the selected sperm cells modified as described in the Spaulding reference (col. 18, lines 14-56), which are not treated in accordance with any embodiment of the invention, but rather involved neat semen into which antibodies were mixed, there is not a single example of actually achieving even a single fertilization of any egg of any kind of female mammal whether in vitro or in vivo to show that frozen selected sperm cells (those with antibodies attached) were fertile upon thawing.

By comparison, as discussed above in appellant's discussion of the breadth of the term "sperm", and as described by the 11 field trials set out in the specification (page 45, line 2) the main objective was to determine fertility of frozen selected sperm processed in

accordance with the various embodiments of the invention. Specification at page 41, starting at line 7. The pregnancy rates in accordance with embodiments of the invention were 49.0% with 203 pregnancies overall. Specification page 45, lines 19-20.

Moreover, the specification provides evidence that these pregnancies were actually accomplished by the selected sperm cells. In those trials with frozen flow sorted selected sperm cells, the subject matter of claim 8, "where the objective was to obtain female offspring, except in trial 8, accuracy was 95%, 83%, 90%, 82%, and 94%. . . respectively. Specification page 45, lines 27-28.

These results evidence a successful test as set out by Salisbury showing that the "frozen selected sperm cells" made in accordance with the invention are fertile. Salisbury at page 442.

"This result surprising because of the well-documented fragility of sperm" as set out in the appellants specification (Specification for example at pages 3, lines 13 -14; page 1, lines 28-31; page2, line 1, page 2, lines 3-4; page 2, lines 15-17; page 6, lines 21-27) and the combination of references provided by the office (Salisbury for example at pages 444, 445, 445, and 446); the scientific understanding prior to the invention as set out in the Offices own combined citations which shows that "the exact nature of total freeze-thaw damage is not fully understood" and that "it is impossible" to generalize one set of conditions for freezing sperm cells to a novel set of circumstances (Salisbury at pages 497 and 499); and the failure of others, including but not limited to those inventors

and investigators in the combination of references provided by the Office, to make the showing first.

The appellants have demonstrated for the first time that pregnancies can be achieved with sperm that has been selected and then frozen. Appellants have provided by comparison to the combination of references provided by the Office, evidence of a benefit not obtained prior to the invention itself, showing that the frozen selected sperm cells which are the subject matter of claim 1 are fertile selected sperm cells capable of fertilizing an egg of a female mammal and achieving pregnancies.

Because appellant has shown an unexpected advantage over the art, appellant respectfully requests that the examiner's rejection of claims 1-27 and 35 be reversed, and the claims allowed as originally recited, or amended to clarify the sperm cells are fertile.

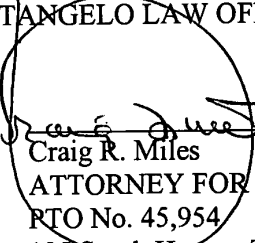
SUMMARY

For the foregoing reasons, it is submitted that the examiner's rejections of claims 1-26 and 35 were erroneous, and reversal of his decision is respectfully requested.

Dated this 7 day of October 2002.

Respectfully Submitted,
SANTANGELO LAW OFFICES, P.C.

By:


Craig R. Miles

ATTORNEY FOR APPLICANT

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APPENDIX A
CLAIMS 1-37 AS ORIGINALLY FILED

1. A method for the cryopreservation of sperm comprising:
 - (a) obtaining a selected sperm sample;
 - (b) cooling said selected sperm sample;
 - (c) isolating sperm from said selected sperm sample to produce isolated sperm;
 - (d) adding final extender to said isolated sperm to produce a suspension of sperm; and
 - (e) freezing said suspension of sperm.
2. The method of Claim 1 wherein said selected sperm sample comprises a portion of the sperm present in a source sample, said portion of sperm selected for a characteristic, and wherein the sperm concentration in the selected sperm sample is lower than in the source sample.
3. The method of Claim 1 wherein said selected sperm sample comprises sex-selected sperm.
4. The method of Claim 1 wherein said selected sperm sample comprises mammalian sperm.

5. The method of Claim 1 wherein said selected sperm sample comprises bovine sperm.
6. The method of Claim 1 wherein said selected sperm sample comprises equine sperm.
7. The method of Claim 1 wherein said selected sperm sample comprises porcine sperm.
8. The method of Claim 1 wherein said selected sperm sample comprises sperm selected by a method from the group consisting of flow cytometry, a magnetic technique, a columnar technique, a gravimetric technique, a biomedical technique, a technique based on motility of sperm, a technique based on an electrical property of sperm, and any combination thereof.
9. The method of Claim 8 wherein sperm have been selected by flow cytometry.
10. The method of Claim 1 wherein cooling is carried out by reducing the temperature of the selected sperm sample to about 5°Celsius.
11. The method of Claim 10 wherein cooling is carried out over a period of about 60 minutes to about 240 minutes.

12. The method of Claim 1 wherein the final extender added to said selected sperm sample each comprise, in addition to cryoprotectant, one or more of the following components: a component that maintains osmolality and buffers pH, an organic substance that reduces cold shock and preserves fertility of sperm, an energy source, a substance that facilitates sperm capacitation, and an antibiotic.
13. The method of Claim 12 wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.
14. The method of Claim 12 wherein said cryoprotectant is selected from the group consisting of glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.
15. The method of Claim 12 wherein said component that maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.
16. The method of Claim 12 wherein said component that maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris{hydroxymethyl}aminomethane, N-Tris]hydroxymethyl]methyl-2-aminoethanesulfonic acid, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

17. The method of Claim 12 wherein said organic substance is selected from the group consisting of egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.
18. The method of Claim 12 wherein said energy source is a monosaccharide selected from the group consisting of glucose, fructose, mannose, and any combination thereof.
19. The method of Claim 12 wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.
20. The method of Claim 1 wherein, after the addition of the final extender the sperm sample and suspension of sperm, respectively, comprise glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.
21. The method of Claim 1 wherein, after the addition of the final extender said sperm sample and suspension of sperm, each comprise glycerol, sodium citrate, egg yolk, and one or more antibiotics.

22. The method of Claim 1 wherein, after the addition of the final extender said sperm sample and suspension of sperm, each comprise glycerol, egg yolk, milk, fructose, and one or more antibiotics.
23. The method of Claim 1 wherein said extender has a pH in the range of about 6.5 to about 7.5.
24. The method of Claim 1 wherein the sperm are isolated from said selected sperm sample by centrifugation.
25. The method of Claim 24 wherein said centrifugation allows for at least about 50% to about 90% recovery of sperm.
26. The method of Claim 1 wherein the concentration of sperm in said suspension prior to freezing is about $1 \times 10^6/\text{ml}$ to about $300 \times 10^6/\text{ml}$.
27. A frozen selected sperm sample comprising portion of the sperm present in a source sample, said portion of sperm selected for a characteristic.
28. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample comprises sex-selected sperm.

29. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample comprises mammalian sperm.
30. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample comprises bovine sperm.
31. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample comprises equine sperm.
32. The frozen selected sperm sample of Claim 29 wherein frozen selected sperm sample comprises porcine sperm.
33. The frozen selected sperm sample of Claim 27 wherein the method used to select said selected sperm sample comprises a technique, a columnar technique, a gravimetric technique, a biochemical technique, a technique based on motility of sperm, a technique based on an electrical property of sperm, and any combination thereof.
34. The frozen selected sperm sample of Claim 33 wherein said frozen selected sperm sample comprises sperm that have been selected by flow cytometry.
35. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample is produced by a method comprising:

- (a) obtaining a selected sperm sample;
- (b) cooling said selected sperm sample;
- (c) isolating sperm from said selected sperm sample to produce isolated sperm;
- (d) adding final extender to said isolated sperm to produce a suspension of sperm; and
- (e) freezing said suspension of sperm.

- 36. A method comprising using the frozen selected sperm sample of Claim 27 for artificial insemination or in vitro fertilization.
- 37. The method of Claim 36 comprising using frozen selected sperm sample for low-dose artificial insemination.

EXHIBIT B

ELECTED CLAIMS IN GROUP I (1-26 AND 35)

1. A method for the cryopreservation of sperm comprising:
 - (a) obtaining a selected sperm sample;
 - (b) cooling said selected sperm sample;
 - (c) isolating sperm from said selected sperm sample to produce isolated sperm;
 - (d) adding final extender to said isolated sperm to produce a suspension of sperm; and
 - (e) freezing said suspension of sperm.
2. The method of Claim 1 wherein said selected sperm sample comprises a portion of the sperm present in a source sample, said portion of sperm selected for a characteristic, and wherein the sperm concentration in the selected sperm sample is lower than in the source sample.
3. The method of Claim 1 wherein said selected sperm sample comprises sex-selected sperm.
4. The method of Claim 1 wherein said selected sperm sample comprises mammalian sperm.

5. The method of Claim 1 wherein said selected sperm sample comprises bovine sperm.
6. The method of Claim 1 wherein said selected sperm sample comprises equine sperm.
7. The method of Claim 1 wherein said selected sperm sample comprises porcine sperm.
8. The method of Claim 1 wherein said selected sperm sample comprises sperm selected by a method from the group consisting of flow cytometry, a magnetic technique, a columnar technique, a gravimetric technique, a biomedical technique, a technique based on motility of sperm, a technique based on an electrical property of sperm, and any combination thereof.
9. The method of Claim 8 wherein sperm have been selected by flow cytometry.
10. The method of Claim 1 wherein cooling is carried out by reducing the temperature of the selected sperm sample to about 5°Celsius.
11. The method of Claim 10 wherein cooling is carried out over a period of about 60 minutes to about 240 minutes.

12. The method of Claim 1 wherein the final extender added to said selected sperm sample each comprise, in addition to cryoprotectant, one or more of the following components: a component that maintains osmolality and buffers pH, an organic substance that reduces cold shock and preserves fertility of sperm, an energy source, a substance that facilitates sperm capacitation, and an antibiotic.
13. The method of Claim 12 wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.
14. The method of Claim 12 wherein said cryoprotectant is selected from the group consisting of glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.
15. The method of Claim 12 wherein said component that maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.
16. The method of Claim 12 wherein said component that maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris{hydroxymethyl}aminomethane, N-Tris]hydroxymethyl]methyl-2-aminoethanesulfonic acid, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

17. The method of Claim 12 wherein said organic substance is selected from the group consisting of egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.
18. The method of Claim 12 wherein said energy source is a monosaccharide selected from the group consisting of glucose, fructose, mannose, and any combination thereof.
19. The method of Claim 12 wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.
20. The method of Claim 1 wherein, after the addition of the final extender the sperm sample and suspension of sperm, respectively, comprise glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.
21. The method of Claim 1 wherein, after the addition of the final extender said sperm sample and suspension of sperm, each comprise glycerol, sodium citrate, egg yolk, and one or more antibiotics.

22. The method of Claim 1 wherein, after the addition of the final extender said sperm sample and suspension of sperm, each comprise glycerol, egg yolk, milk, fructose, and one or more antibiotics.
23. The method of Claim 1 wherein said extender has a pH in the range of about 6.5 to about 7.5.
24. The method of Claim 1 wherein the sperm are isolated from said selected sperm sample by centrifugation.
25. The method of Claim 24 wherein said centrifugation allows for at least about 50% to about 90% recovery of sperm.
26. The method of Claim 1 wherein the concentration of sperm in said suspension prior to freezing is about $1 \times 10^6/\text{ml}$ to about $300 \times 10^6/\text{ml}$.
35. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample is produced by a method comprising:
- (a) obtaining a selected sperm sample;
 - (b) cooling said selected sperm sample;
 - (c) isolating sperm from said selected sperm sample to produce isolated sperm;

- • • •
- (d) adding final extender to said isolated sperm to produce a suspension of sperm; and
 - (e) freezing said suspension of sperm.



Docket No. 22091-701

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Mary B. Zimmerman
Mary B. Zimmerman

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application for:

John L. SCHENK

Serial No.: 09/478,299

Filing Date: January 5, 2000

For: METHOD OF CRYOPRESERVING
SELECTED SPERM CELLS

Examiner: Michael V. Meller

Group Art Unit: 1651

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Commissioner for Patents
Washington, D.C. 20231

Sir:

XY, Inc., the assignee of record of the entire right, title and interest in the above-identified application, hereby revokes all previous Powers of Attorney and appoints Craig Miles, Reg. No. 45,954 whose mailing address is Santangelo Law Offices, P.C., 125 South Howes Street, 3rd Floor, Fort Collins, CO 80521 as its attorney to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

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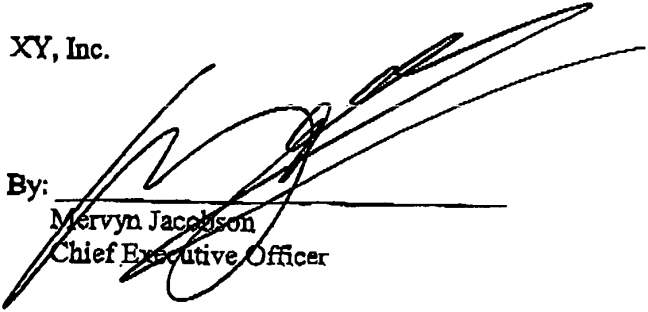
Please direct all telephone calls regarding this application to Craig Miles at (970) 224-3100 and send all correspondence to:

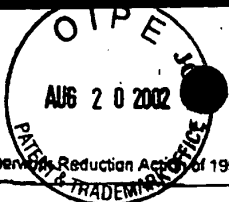
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Respectfully submitted,

XY, Inc.

Dated: AUGUST 2nd, 2002.

By: 
Mervyn Jacobson
Chief Executive Officer



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STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: John L. SCHENK
Application No./Patent No.: 09/478,299 Filed/Issue Date: January 5, 2000

XY, INC., a Corporation

(Name of Assignee)

(Type of Assignee, e.g. corporation, partnership, university, government agency, etc.)

states that it is:

1. ☒ the assignee of the entire right, title and interest; or
2. ☐ an assignee of less than the entire right, title and interest.
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in the patent application/patent identified above by virtue of either:

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The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

August 22, 2000
Date

Mervyn Jacobson

Typed or printed name

[Signature]
Signature

Chief Executive Officer

Title

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09/478,299	01/05/2000	John L. Schenk	22091-701	1509

7590 12/18/2002

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19

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**BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES**

Paper No. 19

Application Number: 09/478,299
Filing Date: January 05, 2000
Appellant(s): SCHENK, JOHN L.

Craig R. Miles
For Appellant

EXAMINER'S ANSWER

This is in response to the appeal brief filed 10/07/2002.

(1) *Real Party in Interest*

A statement identifying the real party in interest is contained in the brief.

(2) *Related Appeals and Interferences*

A statement identifying the related appeals and interferences which will directly affect or be directly affected by or have a bearing on the decision in the pending appeal is contained in the brief.

(3) *Status of Claims*

The statement of the status of the claims contained in the brief is incorrect. A correct statement of the status of the claims is as follows:

On March 7, 2002, appellant appealed the Final rejection of claims 1-26 and 35. Claims 27-34, 36 and 37 were withdrawn from further consideration by the examiner. Appellant noted this in his response filed 6/25/2001.

(4) *Status of Amendments After Final*

The appellant's statement of the status of amendments after final rejection contained in the brief is correct.

(5) Summary of Invention

The summary of invention contained in the brief is correct.

(6) Issues

The appellant's statement of the issues in the brief is substantially correct. The changes are as follows:

The 35 USC 112, second paragraph rejection, was dropped since it was not repeated in the Final rejection.

(7) Grouping of Claims

Appellant's brief includes a statement that claims 1-26 and 35 do not stand or fall together and provides reasons as set forth in 37 CFR 1.192(c)(7) and (c)(8).

(8) Claims Appealed

The copy of the appealed claims contained in the Appendix to the brief is correct.

(9) Prior Art of Record

5,021,244

Spaulding

6-1991

Art Unit: 1654

Salisbury et al., Physiology of Reproduction and Artificial Insemination of Cattle, 2nd Ed., San Francisco: W. H. Freeman, 1978, pages 442-554.

(10) Grounds of Rejection

The following ground(s) of rejection are applicable to the appealed claims:

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 1-26 and 35 stand finally rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. taken with Spaulding.

The claims are drawn to a method for the cryopreservation of sperm as in claims 1 and 35 using as an extender the elected extender containing glycerol and a component that maintains osmolality and buffers pH.

Salisbury teaches cryopreservation of bull semen using different extenders including glycerol (page 497) and a component that maintains osmolality and buffers pH such as sodium citrate, Tris, milk, etc. Salisbury also teaches obtaining the sperm sample cooling it to 5°C for 140 minutes (see page 475-see also pages 463-464 for

additional cooling semen), adding the extender (see page 456, 499), and freezing the suspension of sperm (see pages 471, 494, 495, 503-504). The pH of the extender is 6.5 to 7.0 (page 502 and page 456). See entire reference especially the pages mentioned.

Salisbury does not teach to specifically isolate the sperm using centrifugation and does not teach using flow cytometry to select the sperm sample.

Spaulding teaches to sort sperm for their X or Y characteristic, by flow cytometry and to centrifuge sperm cells to remove seminal plasma proteins, i.e. isolate the sperm, after cooling, see example I.

It would have been obvious to one of ordinary skill in the art at the time the invention was made to use flow cytometry to select sperm for a particular characteristic in the method of Salisbury since Spaulding teaches using flow cytometry to sort X from Y sperm. To centrifuge (isolate) the sperm sample of Salisbury after cooling would have been obvious since Spaulding teaches to remove seminal plasma proteins, i.e. isolate the sperm, after cooling.

Since Salisbury uses bull sperm it also would have been obvious to use other mammalian sperm such as equine and porcine since they are also mammalian sperm and would be expected to work well in the process of Salisbury as well. To use different mammalian sperm is well within the purview of the skilled artisan.

A 50% to 90% recovery of sperm from the centrifugation is obvious since one would want to yield as much of the sperm as possible.

The concentration of sperm in said suspension prior to freezing would be inherent to the suspension of sperm.

The order of the steps of the process of Salisbury might be out of order from the claimed process, but this still is obvious. MPEP 2144.04 IV (C) states that it is *prima facie* obvious to perform in any order steps of a process.

Thus, the claimed subject matter is obvious over the cited references.

(11) Response to Argument

Appellant first argues that the references do not teach any "viable sperm".

This is not well taken since it is clear from the title of Salisbury that he intends to artificially inseminate cattle. Why would one of ordinary skill in the art artificially inseminate with non-viable sperm ? Clearly Salisbury intends for the sperm to be viable.

Spaulding clearly contemplates viable sperm. Spaulding also in his title describes that their invention will increase the probability that offspring will be of a desired sex. Further, on column 5, lines 4-8 of the reference, Spaulding notes that the sex selected semen can be used in fertilizing ova to produce offspring of the desired sex. Clearly, Spaulding also clearly contemplated the use of viable sperm.

Next, appellant argues that there is no motivation in Salisbury to perform the step of "obtaining a selected sperm sample", but Salisbury is clear in stating that the semen is "obtained" and used , see page 450 of Salisbury. Any sperm sample which is

"obtained" is selected. The term "selected" is so broad as to encompass simply obtaining the sperm sample.

Appellant next argues that Salisbury does not disclose any limitation of the invention because he alleges that Salisbury states that it is "impossible to generalize findings" as "one set of conditions may not be acceptable for others". This argument is not understood since Salisbury is clearly teaching that extenders are useful and that there may be variations in extenders but this in and of itself does not negate Salisbury as a pertinent reference in this case.

Appellant then argues that Spaulding does not teach sex selection of sperm but Spaulding is replete with references to such sex selection of sperm, for example see the abstract, col. 2, lines 54-62, col. 4, lines 15-35, col. 5, lines 4-12, example 1. Next appellant argues that Spaulding only teaches a single freezing step but it is clear from the rejection that Salisbury teaches that sperm is well known to be frozen. Spaulding was cited to show the sex selection of the sperm. Salisbury is the primary reference which does show freezing. The fact that Spaulding may only show one freezing step is irrelevant. It still shows a freezing step and such is not the point in question. It is not understood how appellant can construe this to "teach away" from the invention. In fact, since Spaulding was not even relied upon to show a freezing step but appellant points it out, this only supports the arguments that freezing of sperm is known.

Appellant next argues that Spaulding does not teach that sorted sperm cells can be frozen for any purpose. First of all, Salisbury has already established that freezing of

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obtained sperm is known for artificial insemination. Second of all, Salisbury does not have to provide such a teaching because this is not a 35 USC 102 rejection.

In response to appellant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986).

Appellant next argues that equine and porcine sperm were not specifically taught but it is clear in Spaulding that 'mammalian sperm' is taught which would encompass equine and bovine sperm. Equine and bovine are well known mammals and it would have been within the purview of the skilled artisan to use sperm from such mammals since it is clearly desirable to artificially inseminate them as well to produce offspring to use in the farm industry.

Appellant argues that claim 25 has not been met, but when one performs the claimed invention it is inherent that such a broad range of recovery be met since the references teach the same process as appellant.

Appellant then argues that with respect to claims 2 and 26 that Salisbury on page 475 states that the frozen sperm has 20 million sperm per dose (of which cite cannot be found by the examiner on page 475 of Salisbury). Appellant has stated that this is the case and as can be seen 20 million per dose falls in the range of claims 2 and 26. Further, Spaulding teaches that 20 million sperm cells are obtained, see col. 9, lines 15-37.

Appellant next argues that the pH range claimed is not taught but it is clear in Salisbury that it is, see page 455.

Appellant argues that Salisbury only provides teachings as to how to freeze semen which comes with a variety of disclaimers and warnings that "the exact nature of freeze thaw damage is not fully understood", for example. This is the reason why Salisbury uses glycerol. Salisbury clearly wants to produce sperm to artificially inseminate. It is clear how one would make, isolate and freeze the sperm as well as used the sperm for artificial insemination. Appellant once again argues that Spaulding does not teach freezing of the sperm and this was addressed above.

Appellant next states that Salisbury says that dilution of the spermatozoa with simple solutions depresses motility, but as Salisbury also states egg albumin and casein minimize this effect on page 446 of Salisbury.

Appellant next states that there is no disclosure in Spaulding of selecting frozen sperm by flow cytometry. Spaulding does teach flow cytometry for sex selection of sperm (col. 9, lines 15-65) and Salisbury teaches the freezing of the sperm.

Appellant again tries to allege that one extender may not be suitable for one animal as it is for another, but it is clear from Salisbury that the extenders listed do work for many animals in artificial insemination. This argument is not understood since Salisbury is clearly teaching that extenders are useful and that there may be variations in extenders but this in and of itself does not negate Salisbury as a pertinent reference in this case. There are variables but Salisbury makes it clear that the claimed extenders are well known in the art and that to use them is well known.

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Appellant argues also that there is no showing in Spaulding of actually achieving even a single fertilization of any egg, but it is clear on column 5, lines 4-12 of Spaulding that such fertilization is clearly contemplated. Further, it is clear from Salisbury that fertility was achieved, see for example, at page 465, Table 16-10.

For the above reasons, it is believed that the rejections should be sustained.



Respectfully submitted,

MICHAEL V. MELLER
PATENT EXAMINER

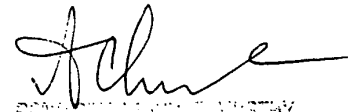
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PATENT EXAMINER



Attorney Docket: XY Frozen RCE
Express Mail No: EV 168806490 US

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IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

n Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

SUBMISSION UNDER 37 C.F.R. § 1.114

Assistant Commissioner for Patents
Box RCE
Washington, DC 20231

The Applicant formally withdraws from appeal of the above-identified application and hereby the following submission under 37 C.F.R. 1.114, as a Request for Continued Examination. This Request for Continued Prosecution responds to the concerns raised in the official communication mailed December 18, 2002. The applicant further submits an Information Disclosure Citation to consolidate the references originally cited by the applicant and those cited by the examiner in prior official communications.

SUBMISSION

Pursuant to 37 C.F.R. §1.121, the applicant submits a clean set of claims as amended. The applicant has consolidated all separate amendments to the claims into a single clean version which is to be construed as a cancellation of all previous versions of the claims with respect to this application. The applicant respectfully requests entry of the clean version of the claims as set forth beginning on the next separate page:

I. 38. A method of freezing sex-selected sperm cells, comprising:

- (a) obtaining sperm cells from a male of a species of mammal;
- (b) sorting said sperm cells based upon sex-type; ?
- (c) cooling sex-selected sperm cells;
- (d) isolating a portion of said sex-selected sperm cells;
- (e) suspending said portion of said sex-selected sperm cells in an extender;
- and
- (f) freezing said sex-selected sperm cells in said extender to provide fertile sex-selected sperm cells upon thawing.

what other species possible sperm.

cls. 60-62

39. The method of freezing sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal comprise bovine sperm cells.

40. The method of freezing sperm cells as described in claim 39, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of bovine sperm cells between about 300,000 and about 3,000,000.

41. The method of freezing sperm cells as described in claim 39, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of bovine sperm cells of no more than about 1,000,000.

42. The method of freezing sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal comprise equine sperm cells.

43. The method of freezing sex-selected sperm cells as described in claim 42, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of equine sperm cells between about 1,000,000 million and about 25,000,000.

44. The method of freezing sex-selected sperm cells as described in claim 42, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of equine sperm cells of no more than about 5,000,000.

45. The method of freezing sex-selected sperm cells as described in claim 38, wherein said step of cooling sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells to about 5°Celsius.

46. The method of freezing sex-selected sperm cells as described in claim 45, wherein said step of reducing the temperature of said sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells over a period of about 60 minutes to about 240 minutes.

47. The method of freezing sex-selected sperm cells as described in claim 38, wherein said extender further comprises a component which maintains osmolality and buffers pH. ①

48. The method of freezing sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof. ② P.

49. The method of freezing sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris[hydroxymethyl]aminomethane, N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof. ③ P.

50. The method of freezing sex-selected sperm cells as described in claim 47, 48, or 49, wherein said extender has a pH in the range of about 6.5 to about 7.5. ④

51. The method of freezing sex-selected sperm cells as described in claim 47, wherein said extender further comprises a cold shock protectant. ⑤

52. The method of freezing sex-selected sperm cells as described in claim 51, wherein said cold shock protectant is selected from the group consisting of egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.

④ sp.

53. The method of freezing sex-selected sperm cells as described in claim 51, wherein said extender further comprises an energy source.

③

54. The method of freezing sex-selected sperm cells as described in claim 53, wherein said energy source is selected from the group consisting of a saccharide, glucose, fructose, mannose, and any combination thereof.

④ sp.

55. The method of freezing sex-selected sperm cells as described in claim 53, wherein said extender further comprises an antibiotic.

④

56. The method of freezing sex-selected sperm cells as described in claim 55, wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.

④ sp.

57. The method of freezing sex-selected sperm cells as described in claim 47, 51, 53, or 55, wherein said extender further comprises a cryoprotectant.

⑤

58. The method of freezing sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.

sp. ④

59. The method of freezing sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.

sp. ④

60. The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which said portion of said sex-selected sperm cells is suspended comprises glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

61. The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which said portion of said sex-selected sperm cells is suspended comprises glycerol, sodium citrate, egg yolk, and one or more antibiotics.

62. The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which said portion of said sex-selected sperm cells is suspended comprises glycerol, egg yolk, milk, fructose, and one or more antibiotics.

63. The method of freezing sex-selected sperm cells as described in claim 38, further comprising the step of equilibrating said portion of said sex-selected sperm cells suspended in said extender prior to freezing over a period of about 1 hour to about 18 hours.

64. The method of freezing sex-selected sperm cells as described in claim 38, further comprising the step of equilibrating said portion of said sex-selected sperm cells suspended in said extender prior to freezing over a period of not greater than 6 hours.

65. A frozen sex-selected sperm sample in accordance with the method of claim 38.

REMARKS

The applicant formally withdraws the appeal pending with respect to this application and files this Request For Continued Prosecution to respond to the concerns raised in the examiner's Answer and present amended claims for allowance.

Appeal Formally Withdrawn. The applicant formally withdraws the appeal pending with respect to application no. 09/48,299. The applicant does not waive any right to a further appeal in this matter, if the claimed subject matter as amended is again finally rejected.

Request For Continued Prosecution. The applicant makes this submission to address the examiner's concerns raised in the Answer mailed December 18, 2002 and respectfully requests examination of the claims as amended.

Formal Cancellation Of Claims. The applicant without prejudice formally cancels claims 1-37. The applicant does not waive any right to have these claims examined without any reduction in breadth in a subsequent continuing application, if desired.

Amendment. The applicant has amended and reordered the sequence of the original claims 1-26 and 35. For clarity purposes the applicant has canceled original claims 1-26 and 35 and set out the amended and reordered claim set as claims 38-65. The applicant respectfully requests entry and examination of claims 38-65 as set out above and provides the following remarks that fully address each concern raised by the Answer mailed on December 18, 2002. The applicant does not believe that the amendments necessitate any further search on the part of the examiner.

As to the concerns under section 103, it should be noted that the claims of the application have been amended to include additional limitations supported by the specification. The applicant has selected these limitations solely to expedite the examination of this application. The applicant believes that these limitations are

allowable based on the examiner's prior completed search and prior communications including the interview of December 18, 2001.

In spite of the amendment, the applicant continues to respectfully disagree on the issue as to whether the previously cited references actually make the claims as originally recited obvious. However, so that the intended legal effect may be properly understood in light of recent case law, all third persons should understand that the proposed amendment is made only as a practicality to permit proceeding efficiently on certain claims as part of a request for continued examination in this immediate application.

The amendments should not be interpreted as an action that in any way surrenders a particular equivalency, surrenders any patent coverage, surrenders any right to patent coverage, restricts the scope of protection intended, or otherwise limits any rights which the applicant may now or hereafter assert. In particular, it should be noted that all rights to pursue the claims as originally recited and to pursue cancelled claims are expressly reserved.

Section 103 Concerns. The applicant provides the following remarks to address the 103 concerns of the examiner raised in the Examiner's Answer mailed December 18, 2002 which are in addition to those provided in Appellant's Brief submitted October 7, 2002. Appellant's Brief attached as Exhibit A; Examiner's Answer attached as Exhibit B.

The examiner first raises the concern that the Salisbury reference teaches "viable sperm". The examiner has improperly broadened the applicant's definition of "selected sperm sample" to include the products taught by Salisbury. "Selected sperm" as defined in the description does not include all manner of "viable sperm" and only includes "sperm that has been subdivided based on presence or absence of a specific characteristic. . . a selected sperm sample is therefore enriched, relative to the source sample, in sperm having the specific characteristic". Specification at page 4, ll. 9-13. For example certain embodiments of the invention include, "sperm selected from a source sample for a

characteristic. . . include frozen sex-selected human, bovine, equine, ovine, elk, or bison. Sex-selection is preferably carried out using flow cytometry. . .” Specification at page 17, ll. 19-25. Salisbury does not teach any aspect of “selected sperm” as defined by the applicant and certainly does not teach any aspect of freezing “selected sperm” as defined by the applicant.

The examiner than raises a concern that “any sperm sample which is obtained is selected” and “Salisbury is clear that the semen is obtained”. Answer at pages 6 and 7. Again, the examiner fails to use explicit definition provided by the applicant which controls the interpretation of “selected” as it is used in the claim. MPEP, § 2106. As such “selected” is not so broad as to encompass simply obtaining the sperm sample.

The examiner further raises a concern that Spaulding “contemplates” viable sperm. Answer at page 6. First, the applicants argument is confined to the limitations set out by original claim 1, element f. which limits sperm to fertile frozen selected sperm cells. The examiner’s citation at col.5, ll. 4-8 does not include any freezing limitation at all. The Spaulding reference simply does not teach “fertile frozen selected sperm”.

As such, the combination of references do not teach the limitation of fertile frozen selected sperm cells nor does the combination suggest that “fertile frozen selected sperm” can be obtained through the combined teachings.

Moreover, the combination of references does not provide any expectation of successfully making the invention of “fertile frozen selected sperm cells”. The Salisbury reference makes this unequivocally clear.

First, that there is a “test” to determine efficacy of any freezing procedure. The test is set out in Salisbury.

“Spermatozoa must be alive to be fertile. . .motility does not guarantee that such cells are fertile. . .the integrity of the genetic material in the sperm chromatin must also be preserved. . .extending media and storage conditions should preserve both functions

but there is no test of semen quality prior to use that can predict how well the genetic material is maintained."

Salisbury, Chapter 16, page 442 (emphasis added).

The importance of this test is emphasized throughout the reference by repetition:

"The end fertility level is the ultimate test of superiority of any semen handling method." Salisbury, Chapter 17, page 532.

"Although the ultimate test of a procedure is fertility (141). . ." Salisbury, Chapter 17, page 534.

The reason fertility testing is the ultimate test is because other measures of sperm cell viability do not prove fertility in practice. For example, "In general, the more rapid thawing rates resulted in better motility and retention of the acrosomal cap (249). These results (202) disagree with the fertility results obtained by Bean (149)." Salisbury, Chapter 17, page 534.

For convenience the applicant has attached the pages from Salisbury containing the above cited references as Exhibit C with the specific language highlighted.

It is clear from the offices own cited references that unless artificial insemination with frozen and subsequently thawed semen results in pregnancies the combination of references do not provide the requisite suggestion, or expectation of success required to make a case of obviousness under the rules. §2141, MPEP.



While the applicant respectfully disagrees with the examiner that the combination of Salisbury and Spaulding makes the original limitations of claim 1 obvious, the applicant for expediency only has amended original claim one (now set out as claim 38) to further include the further limitations of "sorting said sperm cells based sex-type" and "freezing said sex-selected sperm cells in said extender to provide fertile sex-selected sperm cells upon thawing". "Sorting" is clearly defined in the specification as "a selection method carried out using a fluorescence-activated cell sorter" Specification

page 4, ll. 14-15. "Sex-type" is defined as "the type of sex chromosome present in the sperm (i.e., the X or Y chromosome)." Specification at page 3, ll. 29-30.

The combination of Salisbury and Spaulding do not teach the limitation of "sorting" and do not teach the limitation of "fertile sex-selected sperm cells upon thawing".

The applicant believes that the claims as amended are now in condition for allowance and respectfully request that the examiner conduct a telephone interview to address any outstanding issues.

Version With Markings To Show Changes Made. Pursuant to 37 C.F.R. §1.121, the applicant submits a marked up version of the claims to show changes made beginning on the next full page:

VERSION WITH MARKINGS TO SHOW CHANGES MADE

The applicant has canceled claims 1-37 without prejudice.

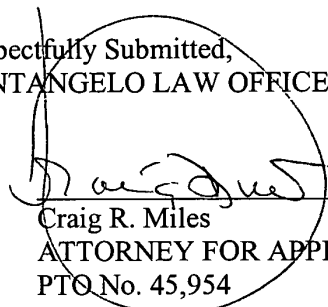
The applicant has added claims 38-65 as set out above.

CONCLUSION

The applicant withdraws the appeal pending in this application. The applicant makes this submission along with Exhibits A-C as a request for continued prosecution. Claims 1-26 and 35 have been canceled and replaced the claim set with claims 38-60. The applicant respectfully requests continued prosecution of claims 38-60.

Dated this 18th day of February 2003.

Respectfully Submitted,
SANTANGELO LAW OFFICES, P.C.

By: 

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(970) 224-3100

Attorney Docket: XY Frozen RCE
Express Mail No: EV 168806490 US

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

n Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

EXHIBIT A

Attorney Docket: XY Frozen RCE
Express Mail No: EV 168806490 US

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

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EXHIBIT B

Attorney Docket: XY Frozen RCE
Express Mail No: EV 168806490 US

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

n Re the Application of: John L. Schenk
Serial Number: 09/478,299
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Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

EXHIBIT C



Express Mail #: EV 168806490 US

PTO/SB/30 (01-03)
Approved for use through 04/30/2003. OMB 0651-0031
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Request For Continued Examination (RCE) Transmittal

Address to:
Commissioner for Patents
Box RCE
Washington, DC 20231

Application Number	09/478,299
Filing Date	January 5, 2000
First Named Inventor	John L. Schenk
Art Unit	1654
Examiner Name	M. Meller
Attorney Docket Number	XY Frozen RCE

TECH CENTER 1600/2900

FEB 24 2003

RECEIVED

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application. Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114

- a. ☐ Previously submitted
- i. ☐ Consider the amendment(s)/reply under 37 CFR 1.116 previously filed on _____
(Any unentered amendment(s) referred to above will be entered).
- ii. ☐ Consider the arguments in the Appeal Brief or Rely Brief previously filed on _____
- iii. ☐ Other _____
- b. ☒ Enclosed
- i. ☒ Amendment/Reply
- ii. ☐ Affidavit(s)/ Declaration(s)
- iii. ☒ Information Disclosure Citation
- iv. ☐ Other _____

2. Miscellaneous

- a. ☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
- b. ☐ Other _____

3. Fees

The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
The Director is hereby authorized to charge the following fees, or credit any overpayments, to

- a. ☐ Deposit Account No. _____
- i. ☒ RCE fee required under 37 CFR 1.17(e) 02/21/2003 AMONDAF1 00000145 09478299
- ii. ☐ Extension of time fee (37 CFR 1.136 and 1.17) 01 FC:2801 375.00 OP
- iii. ☐ Other _____
- b. ☒ Check in the amount of \$ 375.00 enclosed
- c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Name (Print/Type)	Craig R. Miles	Registration No. (Attorney/Agent)	45,954
Signature	<i>[Signature]</i>	Date	February 18, 2003

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner For Patents, Box RCE, Washington, DC 20231, or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Name (Print/Type)	Cheryl A. Swanson	Date	February 18, 2003
Signature	<i>[Signature]</i>		

Page 1 of 2

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, Box RCE, Washington, DC 20231.

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.



Attorney Docket: XY Frozen RCE
Express Mail No: EV 168806490 US

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

n Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

RECEIVED
FEB 24 2003
TECH CENTER 1600/2900

CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.
2. I have this day deposited the attached a Request for Continued Examination under 37 C.F.R. §1.114, (RCE) Transmittal form PTO/SB/30 with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Box RCE Commissioner for Patents, Washington, D.C. 20231.

Dated this 18 day of February, 2003.

Cheryl A. Swanson
Cheryl A. Swanson



02-20-03

RCE/1600 #

Attorney Docket: XY Frozen RCE
Express Mail No: EV 168806490 US

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

n Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

RECEIVED
FEB 24 2003
TECH CENTER 1600/2900

LETTER OF TRANSMITTAL

To: The Commissioner of Patents and Trademarks
BOX RCE
Washington, D.C. 20231

Dear Sir:

Enclosed for filing are the following documents:

1. a Request for Continued Examination under 37 C.F.R. §1.114, (RCE) Transmittal form PTO/SB/30;
2. a Submission Under 37 C.F.R. §1.114 including an amendment and remarks along with Exhibits A, B, and C;
3. an Information Disclosure Citation;
4. this Letter of Transmittal along with a check in the amount of \$375.00 for the RCE fee
5. Certificates of Express Mailing for the above documents and a postcard receipt for return to the undersigned.

Please address all future correspondence to: Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, CO 80521.

Please confirm receipt of the documents by applying your date stamp on the enclosed postcard and returning it to me.

I have this 18 day of February 2003, either myself personally or through my direction of staff at this office, deposited all of the items in the above letter of transmittal with the United States Postal Service as Express Mail, postage prepaid, in an envelope addressed to: Commissioner of Patents and Trademarks, BOX RCE, Washington, D.C. 20231.

Dated this 18 day of February, 2003.

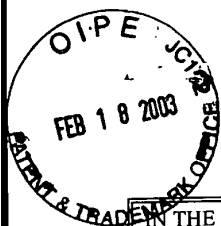
Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By:


Craig R. Miles

ATTORNEY FOR APPLICANT
USPTO Reg. No. 45,954
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

RCE-xmt



21

Express Mail No. EV 168806490 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE CITATION	DOCKET NO. XY Frozen RCE	Serial No. - 09/478,299
	APPLICANT (S) John L. Schenk	
	FILING DATE January 5, 2000	ART UNIT - 1654

I.

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO	DATE	NAME	CLASS	SUBCLASS	FILING DATE
	4,267,268	05/12/81	Nelson, Jr.	435	2	
	4,474,875	10/02/84	Shrimpton	435	2	08/18/80
	4,980,277	12/25/90	Junnila	435	2	
	5,021,244	06/04/91	Spaulding	424	561	05/12/89
	5,135,759	08/04/92	Johnson	424	561	
	5,514,537	05/07/96	Chandler	435	2	

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TECH CENTER 160012900

FEB 24 2003

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II.

FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						Yes	No
	WO 99/33956	08/07/99	US				

III. OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	Amann, Rupert P.; "Cryopreservation of Sperm," Encyclopedia of Reproduction, 1:733-783 (1999)
	Arriola, J. and Foote, R. H.; "Glycerolation and Thawing Effects on Bull Spermatozoa Frozen in Detergent-Treated Egg Yolk and Whole Egg Extenders," J Dairy Sci, 70:1664-1670 (1987)
	Behrman, S. J. and Ackerman, D. R.; "Freeze Preservation of Human Sperm," Am. J. Obstet. and Gynec., 103:5:654-664 (1969)
	Database WPI, Section CH, Week 199920; Derwent Publications Ltd., London, GB; Class B04, AN 1999-229816; XPOO2164167 & BR 9704313 (Alves EW, De Resende Matta M F et al (April 6, 1999 - Abstract)
	DeLeeuw F E et al; "Effects of Various Cryoprotective Agents And Membrane-Stabilizing Compounds on Bull Sperm Membrane Integrity After Cooling and Freezing," (1993), Cryobiology, US, Academic Press Inc.; Vol. 30, No. 1, pp 32-44, XPOO2089949; ISSN 001-2240
	Foote, R. H.; "Buffers and Extenders: What Do They Do? Why Are They Important?" Proc of the 10th NAAB Tech. Conf. on Artificial Insemination and Reproduction, 62-70 (1984)
	Graham, J. K. and Hammerstedt, R. H.; "Differential Effects of Butylated Hydroxytoluene Analogs on Bull Sperm Subjected to Cold-Induced Membrane Stress," Cryobiology, 29:106-117 (1992)

	Hammerstedt, et al., "Cryopreservation of Mammalian Sperm: What We Ask Them to Survive," Journal of Andrology, 11:1:73-88 (190)
	Maxwell, W. and Johnson, L., "Chlortetracycline Analysis of Boar Spermatozoa after Incubation, Flow Cytometric Sorting, Cooling, or Cryopreservation", Molecular Reproduction and Development 46, 1997, pp. 408-418.
	Polge, et al., "Revival of Spermatozoa After Vitrification and Dehydration at Low Temperatures," Nature, 164:666 (1949)
	Pursel, et al., "Effect of Orvus ES Paste on Acrosome Morphology, Motility and Fertilizing Capacity of Frozen-Thawed Boar Sperm," Journal of Animal Science, 47:1:198-202 (1978)
	Salisbury, G.W. and VanDemark, N.L. "Physiology of Reproduction and Artificial Insemination of Cattle." San Francisco: Freeman and Company. pgs. 442-554 (1978)
	Schenk, J.L., "Cryopreservation of flow-sorted bovine spermatozoa", Theriogenology, Vol. 52, 1375-1391 (1999)
	Seidel, G.E., Jr., et al., "Insemination Of Heifers With Very Low Numbers Of Frozen Spermatozoa." , Colorado State University, Fort Collins, Atlantic Breeders Cooperative, Lancaster, PA, DUO Dairy, Loveland, CO, July 1996.
	Stap, et al., "Improving the Resolution of Cryopreserved X- and Y-Sperm During DNA Flow Cytometric Analysis with the Addition of Percoll to Quench the Fluorescence of Dead Sperm; Academic Medical Center, University of Amsterdam (1998)
	Stap, et al., "Improving the Resolution of Cryopreserved X- and Y-Sperm During DNA Flow Cytometric Analysis," (1998), J. Anim. Sci.; Vol. 76, pp. 1896-1902, XP000985715
	Watson, "Recent Development and Concepts in Cryopreservation of Spermatozoa and the assessment of Their Post-thawing Function," Reprod. Fertil. Dev., 7:871-891 (1995)
EXAMINER	DATE CONSIDERED
<i>[Signature]</i>	<i>[Signature]</i> 4/14/03
EXAMINER: Please initial if citation considered, whether or not citation is in conformance with MPEP § 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.	

already
of
record.



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509

7590 03/05/2003
Craig Miles
Santangelo Law Offices P C
125 South Howes Street
3rd Floor
Fort Collins, CO 80521

EXAMINER

MELLER, MICHAEL V 23

ART UNIT	PAPER NUMBER
1654	

DATE MAILED: 03/05/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/478,299	SCHENK, JOHN L.	
	Examiner	Art Unit	
	Michael V. Meller	1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 18 February 2003.
- 2a) ☐ This action is **FINAL**. 2b) ☐ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 38-65 is/are pending in the application.
- 4a) Of the above claim(s) _____ is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☐ Claim(s) _____ is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☒ Claim(s) 38-65 are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
- 11) ☐ The proposed drawing correction filed on _____ is: a) ☐ approved b) ☐ disapproved by the Examiner.
If approved, corrected drawings are required in reply to this Office action.
- 12) ☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

- 13) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
* See the attached detailed Office action for a list of the certified copies not received.
- 14) ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).
a) ☐ The translation of the foreign language provisional application has been received.
- 15) ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)	4) ☐ Interview Summary (PTO-413) Paper No(s). _____
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)	5) ☐ Notice of Informal Patent Application (PTO-152)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____	6) ☐ Other: _____

DETAILED ACTION

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

- I. Claims 38-64, drawn to a method of freezing sex-selected sperm cells, classified in class 424, subclass various.
- II. Claim 65, drawn to a frozen sex-selected sperm sample, classified in class 435, subclass 183.

The inventions are distinct, each from the other because of the following reasons:

Inventions I and II are related as process of making and product made. The inventions are distinct if either or both of the following can be shown: (1) that the process as claimed can be used to make other and materially different product or (2) that the product as claimed can be made by another and materially different process (MPEP § 806.05(f)). In the instant case the product as claimed can be made by a materially distinct process such as genetic engineering.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art as shown by their different classification, restriction for examination purposes as indicated is proper.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art because of their recognized divergent subject matter, restriction for examination purposes as indicated is proper.

This application contains claims directed to the following patentably distinct species of the claimed invention: the many different components (which maintains osmolality and buffers pH) in the extender, cold shock protectant, energy source, antibiotic, cryoprotectant, extender. Applicant is required to enumerate all of the specific components in the composition to be used.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claim 38 is generic.

Applicant is advised that a reply to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 CFR 1.141. If claims are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a).

Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

Applicant is advised that the reply to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed (37 CFR 1.143).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 703-308-4230. The examiner can normally be reached on Monday thru Friday: 9:00am-5:30pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brenda Brumback can be reached on 703-306-3220. The fax phone numbers for the organization where this application or proceeding is assigned are 703-308-0294 for regular communications and 703-308-0294 for After Final communications.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-0196.

Application/Control Number: 09/478,299
Art Unit: 1654

Page 5



Michael V. Meller
Primary Examiner
Art Unit 1654

MVM
March 4, 2003



Express Mail No: EV247367115US
Attorney Docket: XYFroz(Mc)-RCE

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

In Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

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MAR 21 2003

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RESPONSE TO RESTRICTION REQUIREMENT
UNDER 35 U.S.C. § 121

In response to the official action mailed March 5, 2003, the office has required restriction in the above-referenced application. The office has set forth claims 38-64 as Group I, and claim 65 as Group II. A shortened statutory period of thirty days has been set, making a response to this action due on or by April 5, 2003.

The applicant, after review of the office's restriction requirement, hereby elects for further prosecution in the present application the invention of Group I, including claims 38-64, without traverse. The applicant further elects a single species for prosecution if no generic claim is allowable. (X)

Pursuant to 37 C.F.R. § 1.121, the applicant submits a clean set of claims as amended. The applicant has consolidated all separate amendments to the claims into a single clean version which is to be construed as a cancellation of all previous versions of the claims with respect to this application. The applicant respectfully requests entry of the clean version of the claims as set forth beginning on the next separate page:

38. A method of freezing sex-selected sperm cells, comprising:
- (a) obtaining sperm cells from a male of a species of mammal;
 - (b) sorting said sperm cells based upon sex-type;
 - (c) cooling sex-selected sperm cells;
 - (d) isolating a portion of said sex-selected sperm cells;
 - (e) suspending said portion of said sex-selected sperm cells in an extender;
 - (f) freezing said sex-selected sperm cells in said extender; and
 - (g) thawing said sex-selected sperm cells to provide fertile sex-selected sperm cells.
- what other are there?*

B 1
39. The method of freezing sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal are selected from the group consisting of bovine sperm cells and equine sperm cells.

40. The method of freezing sperm cells as described in claim 39, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of bovine sperm cells between about 300,000 and about 3,000,000.

41. The method of freezing sperm cells as described in claim 39, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of bovine sperm cells of no more than about 1,000,000.

42. The method of freezing sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal comprise equine sperm cells.

43. The method of freezing sex-selected sperm cells as described in claim 42, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of equine sperm cells between about 1,000,000 million and about 25,000,000. *E no support*

44. The method of freezing sex-selected sperm cells as described in claim 42, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of equine sperm cells of no more than about 5,000,000. *E no support*

45. The method of freezing sex-selected sperm cells as described in claim 38, wherein said step of cooling sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells to about 5°Celsius.

46. The method of freezing sex-selected sperm cells as described in claim 45, wherein said step of reducing the temperature of said sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells over a period of about 60 minutes to about 240 minutes.

47. The method of freezing sex-selected sperm cells as described in claim 38, wherein said extender further comprises a component which maintains osmolality and buffers pH.

48. The method of freezing sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.

49. The method of freezing sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris[hydroxymethyl]aminomethane, 200mM Tris[hydroxymethyl]aminomethane, 175 mM to 225mM Tris[hydroxymethyl]aminomethane/200 mM Tris[hydroxymethyl]aminomethane/65mM citric acid monohydrate, 175 mM to 225mM Tris[hydroxymethyl]aminomethane/50mM to 70mM citric acid monohydrate, N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/65 mM citric acid monohydrate, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/50mM to 70 mM citric acid monohydrate, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

50. The method of freezing sex-selected sperm cells as described in claim 47, 48, or 49,

wherein said extender has a pH in the range of about 6.5 to about 7.5.

51. The method of freezing sex-selected sperm cells as described in claim 50, wherein said extender further comprises a cold shock protectant.

52. The method of freezing sex-selected sperm cells as described in claim 51, wherein said cold shock protectant is selected from the group consisting of egg yolk, 20% egg yolk, 15% to 25% egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof. *deleted.*

53. The method of freezing sex-selected sperm cells as described in claim 51, wherein said extender further comprises an energy source.

54. The method of freezing sex-selected sperm cells as described in claim 53, wherein said energy source is selected from the group consisting of a saccharide, glucose, fructose, 56 mM fructose, 45mM to 60mM fructose, mannose, and any combination thereof. *deleted.*

55. The method of freezing sex-selected sperm cells as described in claim 53, wherein said extender further comprises an antibiotic.

56. The method of freezing sex-selected sperm cells as described in claim 55, wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.

57. The method of freezing sex-selected sperm cells as described in claim 47, 51, 53, or 55, wherein said extender further comprises a cryoprotectant.

58. The method of freezing sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.

59. The method of freezing sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of glycerol, 6% glycerol, between 5% to 7% glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof. *electd.*

60. The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which said portion of said sex-selected sperm cells is suspended comprises glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

61. The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which said portion of said sex-selected sperm cells is suspended comprises glycerol, sodium citrate, egg yolk, and one or more antibiotics.

62. The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which said portion of said sex-selected sperm cells is suspended comprises glycerol, egg yolk, milk, fructose, and one or more antibiotics.

63. The method of freezing sex-selected sperm cells as described in claim 38, further comprising the step of equilibrating said portion of said sex-selected sperm cells suspended in said extender prior to freezing over a period of about 1 hour to about 18 hours. *what does this mean? how is it done?*

64. The method of freezing sex-selected sperm cells as described in claim 59, further comprising the step of equilibrating said portion of said sex-selected sperm cells suspended in said extender prior to freezing over a period of not greater than 6 hours. *no support*
reads on zero.

65. A frozen sex-selected sperm sample in accordance with the method of claim 38.

66. The method of freezing sex-selected sperm cells as described in claim 39, wherein said step of suspending said portion of said sperm cells in said extender results in a final concentration of said sex-selected sperm cells of greater than 15 million per milliliter of extender. *B2*
inherent.
no support

67. The method of freezing sperm cells as described in claim 66, wherein said step of freezing said sex-selected sperm cells in said extender comprises freezing a number of bovine sperm cells between about 300,000 and about 5,000,000.

REMARKS

The applicant has elected for further prosecution in the present application the invention of Group I, including claims 38-64. The applicant does not waive any right to have the invention of Group II, including claim 65 examined as originally filed without reduction in breadth in a subsequent continuing application.

The applicant has further amended the application to add limitations which enumerate all the specific components in the composition as required by the office in the detailed action.

The office indicates that claim 38 is generic. The applicant elects the following disclosed species for prosecution if no generic claim is finally held allowable as follows:

38/39 (equine sperm cells)/47/49 (200 mM Tris[hydroxymethyl]aminomethane/65mM
citric acid monohydrate)/50/51/52 (20% egg yolk)/53/54 (56mM fructose)/57/59 (6%
glycerol)/64/66/67

see
pg. 456
of Salisbury

VERSION WITH MARKINGS TO SHOW CHANGES MADE

38 (once amended). A method of freezing sex-selected sperm cells, comprising:

- (a) obtaining sperm cells from a male of a species of mammal;
- (b) sorting said sperm cells based upon sex-type;
- (c) cooling sex-selected sperm cells;
- (d) isolating a portion of said sex-selected sperm cells;
- (e) suspending said portion of said sex-selected sperm cells in an extender; [and]
- (f) freezing said sex-selected sperm cells in said extender; and
- (g) thawing said sex-selected sperm cells to provide fertile sex-selected sperm cells [upon thawing].

39 (once amended). The method of freezing sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal are selected from the group consisting of [comprise] bovine sperm cells and equine sperm cells.

40. The method of freezing sperm cells as described in claim 39, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of bovine sperm cells between about 300,000 and about 3,000,000.

41. The method of freezing sperm cells as described in claim 39, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of bovine sperm cells of no more than about 1,000,000.

42. The method of freezing sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal comprise equine sperm cells.

43. The method of freezing sex-selected sperm cells as described in claim 42, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of equine sperm cells between about 1,000,000 million and about 25,000,000.

44. The method of freezing sex-selected sperm cells as described in claim 42, wherein said step of isolating a portion of said sex-selected sperm cells comprises isolating a number of equine sperm cells of no more than about 5,000,000.

45. The method of freezing sex-selected sperm cells as described in claim 38, wherein said step of cooling sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells to about 5°Celsius.

46. The method of freezing sex-selected sperm cells as described in claim 45, wherein said step of reducing the temperature of said sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells over a period of about 60 minutes to about 240 minutes.

47. The method of freezing sex-selected sperm cells as described in claim 38, wherein said extender further comprises a component which maintains osmolality and buffers pH.

48. The method of freezing sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.

49 (once amended). The method of freezing sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris[hydroxymethyl]aminomethane, 200mM Tris[hydroxymethyl]aminomethane, 175 mM to 225mM Tris[hydroxymethyl]aminomethane, 200 mM Tris[hydroxymethyl]aminomethane/65mM citric acid monohydrate, 175 mM to 225mM Tris[hydroxymethyl]aminomethane/50mM to 70mM citric acid monohydrate, N-Tris[[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/65 mM citric acid monohydrate, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/50mM to 70 mM citric acid monohydrate, monosodium glutamate,

milk, HEPES buffered medium, and any combination thereof.

50. The method of freezing sex-selected sperm cells as described in claim 47, 48, or 49, wherein said extender has a pH in the range of about 6.5 to about 7.5.

51 (once amended). The method of freezing sex-selected sperm cells as described in claim [47] 50, wherein said extender further comprises a cold shock protectant.

52 (once amended). The method of freezing sex-selected sperm cells as described in claim 51, wherein said cold shock protectant is selected from the group consisting of egg yolk, 20% egg yolk, 15% to 25% egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.

53. The method of freezing sex-selected sperm cells as described in claim 51, wherein said extender further comprises an energy source.

54 (once amended). The method of freezing sex-selected sperm cells as described in claim 53, wherein said energy source is selected from the group consisting of a saccharide, glucose, fructose, 56 mM fructose, 45mM to 60mM fructose, mannose, and any combination thereof.

55. The method of freezing sex-selected sperm cells as described in claim 53, wherein said extender further comprises an antibiotic.

56. The method of freezing sex-selected sperm cells as described in claim 55, wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.

57. The method of freezing sex-selected sperm cells as described in claim 47, 51, 53, or 55, wherein said extender further comprises a cryoprotectant.

58. The method of freezing sex-selected sperm cells as described in claim 57, wherein said

cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.

59 (once amended). The method of freezing sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of glycerol, 6% glycerol, between 5% to 7% glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.

60. The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which said portion of said sex-selected sperm cells is suspended comprises glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

61. The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which said portion of said sex-selected sperm cells is suspended comprises glycerol, sodium citrate, egg yolk, and one or more antibiotics.

62. The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which said portion of said sex-selected sperm cells is suspended comprises glycerol, egg yolk, milk, fructose, and one or more antibiotics.

63. The method of freezing sex-selected sperm cells as described in claim 38, further comprising the step of equilibrating said portion of said sex-selected sperm cells suspended in said extender prior to freezing over a period of about 1 hour to about 18 hours.

64. The method of freezing sex-selected sperm cells as described in claim [38] 59, further comprising the step of equilibrating said portion of said sex-selected sperm cells suspended in said extender prior to freezing over a period of not greater than 6 hours.

65. A frozen sex-selected sperm sample in accordance with the method of claim 38.

(newly added) -- 66. The method of freezing sex-selected sperm cells as described in claim 39, wherein said step of suspending said portion of said sperm cells in said extender results in a final concentration of said sex-selected sperm cells of greater than 15 million per milliliter of extender.

67. The method of freezing sperm cells as described in claim 66, wherein said step of freezing said sex-selected sperm cells in said extender comprises freezing a number of bovine sperm cells between about 300,000 and about 5,000,000.--

Dated this 17th day of March, 2003.

Respectfully Submitted,
SANTANGELO Law Offices, P.C.

By:


Craig R. Miles

ATTORNEY FOR APPLICANT
PTO No. 45,954
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100



03-19-03

1654

24B
JP

Express Mail No: EV247367115US
Attorney Docket: XYFroz(Mc)-RCE

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

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MAR 21 2003

TECH CENTER 1600/2900

In Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

LETTER OF TRANSMITTAL

The Assistant Commissioner of Patents
Washington, D.C. 20231

Dear Sir:

In response to the Office Action, dated March 5, 2003, enclosed please find the following documents:

1. a Response to Restriction Requirement Under 35 U.S.C. §121;
3. this Letter of Transmittal;
4. Certificates of Express Mailing for each document; and
5. a postcard receipt for return to the undersigned.

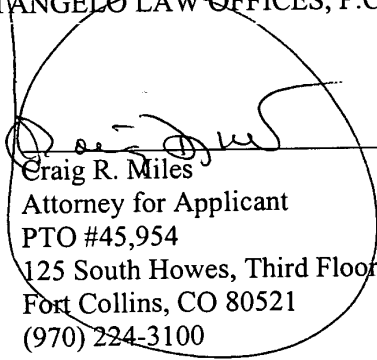
Please address all future correspondence to:

**Santangelo Law Offices, P.C.
125 South Howes, Third Floor
Fort Collins, CO 80521.**

I have this 17th day of March, 2003, either myself personally or through my direction of staff at this office, deposited all of the items in the above letter of transmittal with the United States Postal Service as Express Mail, postage prepaid, in an envelope addressed to: The Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated this 17th day of March, 2003.

Respectfully Submitted,
SANTANGELO LAW OFFICES, P.C.

By: 

Craig R. Miles
Attorney for Applicant
PTO #45,954
125 South Howes, Third Floor
Fort Collins, CO 80521
(970) 224-3100



Express Mail No: EV247367115US
Attorney Docket: XYFroz(Mc)-RCE

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

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MAR 21 2003

TECH CENTER 1600/2900

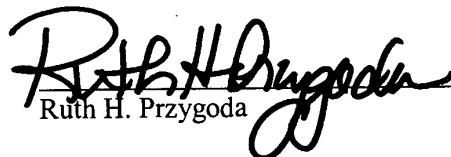
In Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

CERTIFICATE OF EXPRESS MAILING

I, Ruth H. Przygoda, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.
2. I have this day deposited the attached Letter of Transmittal with the United States Postal Service as Express Mail, postage prepaid, in an envelope addressed to: The Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated this 17th day of March, 2003.


Ruth H. Przygoda



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509

7590 04/18/2003

Craig Miles
Santangelo Law Offices P C
125 South Howes Street
3rd Floor
Fort Collins, CO 80521

EXAMINER

MELLER, MICHAEL V

ART UNIT	PAPER NUMBER

1654

DATE MAILED: 04/18/2003

25

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary

Application No.

09/478,299

Applicant(s)

SCHENK, JOHN L.

Examiner

Michael V. Meller

Art Unit

1654

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 17 March 2003.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 38-67 is/are pending in the application.
- 4a) Of the above claim(s) 40, 41, 55, 56, 60-62, 65 and 67 is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 38,39,42-54,57-59,63,64 and 66 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
- 11) ☐ The proposed drawing correction filed on _____ is: a) ☐ approved b) ☐ disapproved by the Examiner.
If approved, corrected drawings are required in reply to this Office action.
- 12) ☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

- 13) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
* See the attached detailed Office action for a list of the certified copies not received.
- 14) ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).
a) ☐ The translation of the foreign language provisional application has been received.
- 15) ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

- 1) ☐ Notice of References Cited (PTO-892) 4) ☐ Interview Summary (PTO-413) Paper No(s). _____
- 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948) 5) ☐ Notice of Informal Patent Application (PTO-152)
- 3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ 6) ☐ Other: _____

DETAILED ACTION

Election/Restrictions

Applicant's election without traverse of Group I, claims 38-64 and the species as noted on page 7 of the response filed 3/17/2003 in Paper No. 24 is acknowledged.

Claims 40, 41, 55, 56, 60-62, 65 and 67 are withdrawn from further consideration by the examiner as being drawn to a non-elected invention.

The restriction requirement is still deemed to be proper and is therefore made FINAL.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 43, 44, 63, 64, 66 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

There is no support in the instant specification for the ranges of sperm cells claimed. The range of 1 hour to 18 hours in claim 63 has no support in the specification.

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Claim 64 finds no support of the time claimed. Claim 66 also finds no support for the number of sperm cells.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 38, 39, 42-54, 57-59, 63, 64, 66 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 38 is confusing since it is not clear how one could obtain sperm cells from another source besides a male when obtaining mammalian sperm. Further, it is unclear how one would equilibrate the portion of the sperm cells in claim 63 and claim 64. What is there to equilibrate ? How is this done ?

It is also not clear how "sex-selected sperm cells" in step c) refers back to the cells in step b).

Also, what is meant by " a portion" ? This term is confusing ? A portion of a sperm cell ? How would one know how much this is ?

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and

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the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 38, 39, 42-54, 57-59, 63, 64, 66 are rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. taken with Spaulding and Shrimpton.

This rejection is of record. The claims are similar to the previous claims. Applicant has elected a specific type of extender and those ingredients are all taught by the references as being well known in the art. It would have been simply the choice of the artisan in an effort to optimize the results to use the extender claimed and since the components are all well known to be used in such a process.

Shrimpton is cited to show that it is well known in sex selection of sperm cells to use horse cells (equine).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 703-308-4230. The examiner can normally be reached on Monday thru Friday: 9:00am-5:30pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brenda Brumback can be reached on 703-306-3220. The fax phone numbers for the organization where this application or proceeding is assigned are 703-308-0294 for regular communications and 703-308-0294 for After Final communications.

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Page 5

Art Unit: 1654

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-0196.

A handwritten signature in black ink, appearing to read 'M V Meller', followed by a horizontal line.

Michael V. Meller
Primary Examiner
Art Unit 1654

MVM
April 14, 2003

Facsimile Number: 703-872-9306

Attorney Docket: 22091-701

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

n Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

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OCT 20 2003

OFFICIAL

RESPONSE and REQUEST for RECONSIDERATION (37 C.F.R. § 1.111)

Mailed to:

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

This amendment and request for reconsideration is in response to the Examiner's Office Action communication (Paper No. 25) mailed April 18, 2003. In that action, the Examiner acknowledged restriction of Claims 40-41, 55-56, 60-62, 65, and 67; questioned issues as to claims 38-39, 42-54, 57-59, 63-64, and 66 under 35 U.S.C. § 112; and expressed concerns with regard to all claims in view of certain references under 35 U.S.C. §103. To respond to all issues, this amendment and request for reconsideration is submitted. This response is made in accordance with 37 C.F.R. 1.121 as amended using the format with each heading beginning on a separate page as follows:

1. Amendments to the Claims are reflected in the listing of all claims beginning on separate page 3 of this response; and
2. Remarks begin on page 9 of this response.

In accordance with the amendment directions provided in 37 CFR 1.121 as recently changed, please amend claims 38, 40-1, 43-44, 60-64, and 66. Each amendment is believed to have been made in accordance with Rule 121, however, should any unintended informality exist, it is requested that the undersigned be contacted by telephone so that it may be resolved as expediently as possible.

Amendments to the claims:

The following listing of claims should be entered to replace all prior listings of claims in the application. In accordance with Rule 121, the status of each claim is indicated parenthetically. In this regard, please note that the brackets in claims 49 and 60 are intended to be part of the claim and do not indicate a deletion. As can be seen, in this listing, claims 38, 40-41, 43-44, 60-64, and 66 have been amended, and claims 39, 45-54, and 57-59 remain unchanged. Claims 38-39, 42-54, 57-59, 63-64, and 66 remain in the application.

38. (Currently amended)

A method of freezing sex-selected sperm cells, comprising:

- (a) obtaining sperm cells ~~from a male~~ of a species of mammal;
- (b) sorting said sperm cells based upon sex-type using flow cytometry to create a collection of sex-selected sperm cells;
- (c) cooling said sex-selected sperm cells;
- (d) isolating ~~a portion of~~ at least some of said sex-selected sperm cells to create a collection of isolated sex-selected sperm cells;
- (e) suspending at least some of said ~~portion of said isolated~~ sex-selected sperm cells in an extender;
- (f) freezing said isolated sex-selected sperm cells in said extender; and
- (g) thawing said isolated sex-selected sperm cells to provide fertile sex-selected sperm cells.

39. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal are selected from the group consisting of bovine sperm cells and equine sperm cells.

40. (Withdrawn)

The method of freezing sperm cells as described in claim 39, wherein said step of isolating ~~a portion~~ at least some of said sex-selected sperm cells comprises isolating a number of bovine sperm cells between about 300,000 and about 3,000,000.

41. (Withdrawn)

The method of freezing sperm cells as described in claim 39, wherein said step of isolating ~~a portion~~ at least some of said sex-selected sperm cells comprises isolating a number of bovine sperm cells of no more than about 1,000,000.

42. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal comprise equine sperm cells.

43. (Currently amended)

The method of freezing sex-selected sperm cells as described in claim 42, wherein said step of isolating ~~a portion~~ at least some of said sex-selected sperm cells comprises isolating a number of equine sperm cells between about 1,000,000 million and about 25,000,000.

44. (Currently amended)

The method of freezing sex-selected sperm cells as described in claim 42, wherein said step of isolating ~~a portion~~ at least some of said sex-selected sperm cells comprises isolating a number of equine sperm cells of no more than about 5,000,000.

45. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 38, wherein said step of cooling sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells to about 5°Celsius.

46. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 45, wherein said step of reducing the temperature of said sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells over a period of about 60 minutes to about 240 minutes.

47. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 38, wherein said extender further comprises a component which maintains osmolality and buffers pH.

48. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.

49. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris[hydroxymethyl]aminomethane, 200mM Tris[hydroxymethyl]aminomethane, 175 mM to 225mM Tris[hydroxymethyl]aminomethane, 200 mM Tris[hydroxymethyl]aminomethane/65mM citric acid monohydrate, 175 mM to 225mM Tris[hydroxymethyl]aminomethane/50mM to 70mM citric acid monohydrate, N-Tris [hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/65 mM citric acid monohydrate, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/50mM to 70 mM citric acid monohydrate, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

50. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 47, 48, or 49, wherein said extender has a pH in the range of about 6.5 to about 7.5.

51. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 50, wherein said extender further comprises a cold shock protectant.
52. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 51, wherein said cold shock protectant is selected from the group consisting of egg yolk, 20% egg yolk, 15% to 25% egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.
53. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 51, wherein said extender further comprises an energy source.
54. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 53, wherein said energy source is selected from the group consisting of a saccharide, glucose, fructose, 56 mM fructose, 45mM to 60mM fructose, mannose, and any combination thereof.
55. (Withdrawn)
The method of freezing sex-selected sperm cells as described in claim 53, wherein said extender further comprises an antibiotic.
56. (Withdrawn)
The method of freezing sex-selected sperm cells as described in claim 55, wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, lincospectin, spectinomycin, penicillin, streptomycin, and any combination thereof.
57. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 47, 51, 53, or 55, wherein said extender further comprises a cryoprotectant.

58. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.

59. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of glycerol, 6% glycerol, between 5% to 7% glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.

60. (Withdrawn)

The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which ~~said portion~~ at least some of said sex-selected sperm ~~cells is~~ cells are suspended comprises glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

61. (Withdrawn)

The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which ~~said portion~~ at least some of said sex-selected sperm ~~cells is~~ cells are suspended comprises glycerol, sodium citrate, egg yolk, and one or more antibiotics.

62. (Withdrawn)

The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which ~~said portion~~ at least some of said sex-selected sperm ~~cells is~~ cells are suspended comprises glycerol, egg yolk, milk, fructose, and one or more antibiotics.

63. (Currently amended)

The method of freezing sex-selected sperm cells as described in claim 38, further comprising the step of equilibrating ~~said portion~~ at least some of said sex-selected sperm

cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to freezing over a period of about 1 hour to about 18 hours.

64. (Currently amended)

The method of freezing sex-selected sperm cells as described in claim 59, further comprising the step of equilibrating ~~said portion~~ at least some of said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to freezing over a period of not greater than 6 hours.

65. (Withdrawn)

A frozen sex-selected sperm sample in accordance with the method of claim 38.

66. (Currently amended)

The method of freezing sex-selected sperm cells as described in claim 39, wherein said step of suspending ~~said portion~~ at least some of said sperm cells in said extender results in a final concentration of said sex-selected sperm cells of greater than ~~15 million~~ 10 million per milliliter of extender.

67. (Withdrawn)

The method of freezing sperm cells as described in claim 66, wherein said step of freezing said sex-selected sperm cells in said extender comprises freezing a number of bovine sperm cells between about 300,000 and about 5,000,000.

REMARKS

Amendments to the claims have been made to refine claim language. In making the amendments, it should be understood that the amendments are to be interpreted in a manner that avoids, where applicable, any admission or estoppel, generally, negatively affecting the scope of protection provided by the disclosure and claims of the present application, and also in a manner that avoids prosecution history estoppel, limitation of the scope of equivalences, or the like.

As to the issues raised by the examiner, it should be understood that support does exist in the specification for the current and previous amendments. As to the current amendments, support concerns are addressed by highlighting areas in specification supporting the following claim aspects:

In claim 63 as amended, the aspect of: "equilibrating at least some of said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to freezing over a period of about 1 hour to about 18 hours" is explicitly discussed in paragraph 76 (with numbering as indicated in the publication of the application), as shown on page 17, line 5 of the original specification where it is stated:

Prior to freezing, the sperm are generally allowed to equilibrate at about 5.degree. C. Preferably, the sperm are allowed to equilibrate for a period in the range of **about 1 hour to about 18 hours**, more preferably between about 3 hours and about 18 hours, and most preferably between about 3 hours and about 6 hours (see Example 2). (Emphasis added.)

From this excerpt, it is clear that the time limitation now posed, namely, the "period of about 1 hour to about 18 hours" is not new matter and was originally discussed in the application.

In claim 64 as amended, the aspect of: "equilibrating at least some of said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to freezing over a period of not greater than 6 hours" is also addressed in the specification. First, paragraph 76 (quoted above) of the original specification discusses the aspect of equilibrating for between about 3 hours and about 6 hours. Table 4 also shows data that a person of ordinary skill in the art would readily understand as demonstrating that equilibration for a period of not greater than six hours yields higher motility percentages. Specifically, reviewing each of the 0h, 1h, and 2h post

thaw assessment times shown in the table, it is evident that motility has a significant drop when equilibration occurs for more than six hours. In the 0h situation motility dropped by 15% (35% motile vs. 41% motile for the shorter equilibrations times). Using the 1h time of assessment, motility similarly dropped by 16% (31% motile vs. 37% motile for the shorter equilibration time). Further, even using the 2h time of assessment, motility dropped by a significant 33% (12% motile vs. 18% motile for the shorter equilibration time). In addition to this data, there is additional discussed in paragraph 114 and 115 (again, with numbering as indicated in the publication of the application), as shown on page 27, line 16 of the original specification where it is stated:

6. Conclusion. The results indicated no differences in post-thaw sperm motility between 3 and 6 h of total equilibration time at 5.degree. C., but there was a significant decline in sperm motility following 18 h of equilibration at 5.degree. C. before freezing. The 3- to 6-h range permits pooling 2 consecutive 3-h sorting batches for freezing sperm without decreasing post-thaw motility.

As the bull by equilibration-time interaction was not significant, 3 to 6 h equilibration was adequate, with the caveat that only 4 bulls were used. The optimum equilibration time for a minority of bulls is expected to be >6 h.

This excerpt makes it clear that only a "minority of bulls" would be expected to have an optimum equilibration time of "greater than 6 hours". From this, a person of ordinary skill in the art would easily understand that, therefore, the majority of bulls are expected to have a equilibration time of "not greater than 6 hours" as now set forth in the claim. Thus it is clear that the time limitation now posed, namely, the period of "not greater than 6 hours" is not new matter and is supported by the original application.

In claim 66, as amended, the aspect of: "suspending at least some of said sperm cells in said extender results in a final concentration of said sex-selected sperm cells of greater than 10 million per milliliter of extender" is explicitly discussed in paragraph 88 (with numbering as indicated in the publication of the application), as shown on page 20, line 9 of the original specification where it is stated:

5. Results. Data for non-washed sperm (Table 1) revealed (log) linear relationships ($P < 0.01$) for both incubation times. Percentages of motile sperm increased as sperm concentration increased from $1.25 \times 10^6/\text{ml}$ to $10 \times 10^6/\text{ml}$, but there was little difference thereafter. The cubic term was significant ($P < 0.05$) for 24-h and marginally significant ($P < 0.1$) for 48-h incubations. There was a bull effect ($P < 0.01$) at both times. (Emphasis added.)

Table 1 as shown on page 22, also shows data that would indicate to a person of ordinary skill in the art that concentrations of greater than 10 million per milliliter are significant to achieving higher motility percentages. Specifically, reviewing the data, it is evident that motility has a significant drop when concentrations are less than 10 million per milliliter. Most notably, in the 48h incubation situation, motility drops by 26% when concentration is below 10 million per milliliter (31% motile vs. 42% motile for the lower concentration value). From these disclosures in the original specification, it is clear that the concentration limit now posed, namely, the "greater than 10 million per milliliter" concentration value is not new matter and was originally discussed in the application. [Note that the earlier 15 million/ml limit is also supported in the original specification, however, this limit is not desired as the claim limitation at this time.]

In addition to the request for the location of the discussions mentioned above for new matter concerns, the action also raised clarity concerns. Each of these has been addressed by amending the language to clarify that which is intended. Specifically, claim 38 has been amended to remove the term "male" as questioned, to resolve the antecedent basis of "sex-selected sperm cells", and to eliminate the potentially confusing "portion" language. Similarly, the questioning of the term "equilibrate" has been clarified in claims 63 and 64, to indicate that the equilibration is to a cooler, non-freezing temperature for a period of time.

Finally, as to the obviousness concerns, this application has been amended to specify the particular type of sorting is the type using flow cytometry. This particular type of sorting is significant because one would usually prefer a sorting method that preserves sperm viability. The challenging conditions (for sperm) such as exist in flow cytometry sorting would usually be avoided, but instead through this invention, these conditions can be overcome. Specifically, the original application beginning in paragraph 38 (numbering as indicated in the publication of the application) shown on page 6, line 20, states:

The selection method used to obtain the selected sperm sample is preferably one that preserves sperm viability. Because of the relative fragility of sperm, normal flow cytometry techniques should generally be modified for sorting sperm. More specifically, the flow cytometry entails staining, dilution, and interrogation of cells with light. All of these steps represent stresses that can reduce sperm viability. The

sensitivity of sperm to these stresses can vary between species and even between individuals within species. Such sensitivities have either been documented or can readily be determined by empirical studies, such as those described in Examples 1-5.

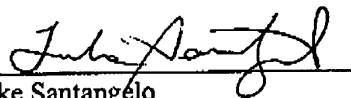
This application highlights the challenges of using flow cytometry for the separation task and also highlights how the combination of freezing with flow cytometry sorting has been overcome. Importantly, none of the references discuss the unique combination of flow cytometry with a freezing process as now possible. Thus it is respectfully submitted that the claims as amended are not obvious in view of the cited references. Finally, as to this and all amendments, it should be understood that this amendment is made without prejudice and with the express reservation of the right to pursue claims without this limitation in this or other applications.

CONCLUSION

The claims have been amended to address matters of form as requested in the action. Claims 40-41, 55-56, 60-62, 65, and 67 have been withdrawn. Claims 38-39, 42-54, 57-59, 63-64, and 66 remain in the application. An early consideration of the present application is earnestly requested.

Dated this 17th day of October, 2003.

Respectfully submitted,



Luke Santangelo
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(970) 224-3100

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FACSIMILE TRANSMISSION

This message is intended only for the use of the individual or entity to which it is addressed, and may contain information that is privileged, confidential and exempt from disclosure under applicable law.

If the reader of this message is not the intended recipient, or the employee or agent responsible for delivering the message to the intended recipient, you are hereby notified that any dissemination, distribution or copying of this communication is strictly prohibited. If you have received this communication in error, please notify us immediately by telephone, and return the original message to us at the above address via the u.s. postal service. Thank you.

TO: Examiner Michael V. Meller, Group Art Unit 1654
FAX: 703-872-9306 **TELEPHONE:** 703-308-4230
FROM: Luke Santangelo, PTO Reg. No. 31,997
DATE: October 17, 2003
TOTAL PAGES: 19 (including this cover page) X Facsimile Filing

LETTER OF FACSIMILE TRANSMITTAL

First Named Inventor: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
For: Method of Cryopreserving Selected Sperm Cells
Assignee: XY, Inc.
Attorney Docket No.: 22091-701

Enclosed for filing in the above application please find the following:

1. a Petition for Extension of Time Under 37 C.F.R. §1.136(a) (2 pages);
2. a Response and Request for Reconsideration (37 C.F.R. § 1.111) (12 pages);
3. a Credit Card Payment Form authorized in the amount of \$950.00 for the 3-month extension of time fee (1 page);
4. Notification of Change of Small Entity Status (1 page);
5. this Letter of Transmittal (2 pages); and
6. a Certificate of Facsimile Transmittal (1 page).

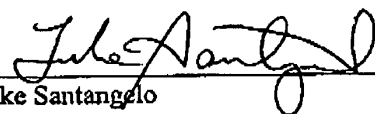
Please confirm receipt of the documents by return facsimile indicating the date of receipt and application number.

I have this 17th day of October 2003, either myself personally or through my direction of staff at this office, forwarded all of the items in the above Letter of Transmittal via Facsimile Number 703-872-9306, Attention: Examiner Michael V. Meller, Group Art Unit 1654.

Dated this 17th day of October, 2003.

Respectfully Submitted,
SANTANGELO LAW OFFICES, P.C.

By:



Luke Santangelo
Attorney for Applicant
PTO #31,997
125 South Howes, Third Floor
Fort Collins, CO 80521
(970) 224-3100

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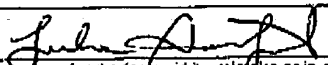
PTO-2038 (02-2000)

Approved for use through 01/31/2003. OMB 0651-0043

United States Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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Refund Policy: The Office may refund a fee paid by mistake or in excess of that required. A change of purpose after the payment of a fee will not entitle a party to a refund of such fee. The Office will not refund amounts of twenty-five dollars or less unless a refund is specifically requested, and will not notify the payor of such amounts (37 CFR 1.26). Refund of a fee paid by credit card will be via credit to the credit card account. Service Charge: There is a 50.00 service charge for processing each payment refused (including a check returned "unpaid") or charged back by a financial institution (37 CFR 1.21(m)).			
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Request and Payment Information			
Description of Request and Payment Information:			
3-Month Extension of Time Fee \$950.00			
Patent Fee	Patent Maintenance Fee	Trademark Fee	Other Fee
Application No.	Application No.	Serial No.	IDON Customer No.
09/478,299			
Patent No.	Patent No.	Registration No.	
Attorney Docket No.		Identify or Describe Mark	
22091-701			

If the cardholder includes a credit card number on any form or document other than the Credit Card Payment Form, the United States Patent & Trademark Office will not be liable in the event that the credit card number becomes public knowledge.

Received from <9702243175> at 10/17/03 5:03:17 PM [Eastern Daylight Time]

Facsimile Number: 703-872-9306

Attorney Docket: 22091-701

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

n Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

NOTIFICATION OF CHANGE OF SMALL ENTITY STATUS

Dear Sir:

Pursuant to 37 C.F.R. 1.27(g)2 this document hereby notifies the Office that small entity status as previously established in the above referenced matter is no longer appropriate.

This notification of change in status is filed prior to or at the time of paying any issue, maintenance, or other fees after the change in status occurred. As required by the rule, this notification is signed by the attorney of record acting in a representative capacity pursuant to 37 C.F.R. 1.27(g)2.

JS
Dated this 17 day of October, 2003.

Respectfully Submitted,
SANTANGELO LAW OFFICES, P.C.

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OCT 20 2003

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By:

Luke Santangelo

Luke Santangelo
USPTO Reg. No. 31,997
125 South Howes, 3rd Floor
Fort Collins, Colorado 80521
(970)224-3100

Facsimile Number: 703-872-9306

Attorney Docket: 22091-701

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

n Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

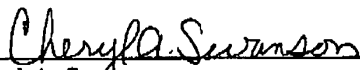
CERTIFICATE OF FACSIMILE TRANSMISSION

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day forwarded the foregoing Letter of Facsimile Transmission; Notification of Change of Small Entity Status; a Petition for Extension of Time Under 37 C.F.R. §1.136(a); a Response and Request for Reconsideration (37 C.F.R. § 1.111), a Credit Card Payment Form authorized in the amount of \$950.00 for the 3-month extension of time fee, and this Certificate of Facsimile Transmission via facsimile number 703-872-9306, addressed to Examiner Michael Meller, Group Art Unit 1654.

Dated this 12th day of October, 2003.


Cheryl A. Swanson

Facsimile Number: 703-872-9306

Attorney Docket: 22091-701

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

n Re the Application of: John L. Schenk
Serial Number: 09/478,299
Filed: January 5, 2000
Parent Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.

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PETITION FOR EXTENSION OF TIME UNDER 37 C.F.R. §1.136(a)

Mail Stop 1654
Facsimile Number 703-872-9306
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Dear Sir:

On behalf of the applicant, the undersigned attorney hereby requests an extension of time pursuant to 37 C.F.R. §1.136(a). This request is submitted in relation to a response due to the office action mailed April 18, 2003, having a shortened statutory period for response of three months. A response to this action on the merits would otherwise be due July 18, 2003; however, it is requested that this time period be extended for three months to and including October 18, 2003. This extension will thereby encompass the statutory period of six months pursuant to 35 U.S.C. §133. Pursuant to 37 C.F.R. §1.17, an extension fee of \$950.00 is submitted with this Petition. In the event the fee has

been inappropriately calculated, please allow the maximum extension possible and inform me at your earliest convenience so that any deficiency or error can be corrected immediately.

Dated this 17th day of October, 2003

Respectfully submitted,

SANTANGELO LAW OFFICES, P.C.

By:


Luke Santangelo
Attorney For Applicant
PTO No. 31,997
315 West Oak Street, #701
Fort Collins, CO 80521
(303) 224-3100

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Interview Summary	Application No.	Applicant(s)	
	09/478,299	SCHENK, JOHN L.	
	Examiner	Art Unit	
	Michael V. Meller	1654	

All participants (applicant, applicant's representative, PTO personnel):

(1) Michael V. Meller. (3) _____

(2) Luke Santangelo. (4) _____

Date of Interview: 29 October 2003.

Type: a) ☐ Telephonic b) ☐ Video Conference
c) ☒ Personal [copy given to: 1) ☐ applicant 2) ☒ applicant's representative]

Exhibit shown or demonstration conducted: d) ☐ Yes e) ☒ No.
If Yes, brief description: _____.

Claim(s) discussed: All pending.

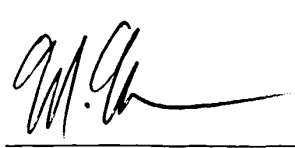
Identification of prior art discussed: All of record.

Agreement with respect to the claims f) ☐ was reached. g) ☒ was not reached. h) ☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: Discussed the issues raised by claim 66 and applicants will submit a supplemental response.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.



Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

Examiner's signature, if required

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record

A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews

Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR § 1.2 Business to be transacted in writing.

All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:

- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

- 1) A brief description of the nature of any exhibit shown or any demonstration conducted,
- 2) an identification of the claims discussed,
- 3) an identification of the specific prior art discussed,
- 4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
- 5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
- 6) a general indication of any other pertinent matters discussed, and
- 7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509
7590	12/24/2003			
Craig Miles Santangelo Law Offices P C 125 South Howes Street 3rd Floor Fort Collins, CO 80521			EXAMINER MELLER, MICHAEL V	
			ART UNIT 1654	PAPER NUMBER

DATE MAILED: 12/24/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.		Applicant(s)	
	09/478,299		SCHENK, JOHN L.	
	Examiner		Art Unit	
	Michael V. Meller		1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on 10/20/2003.

2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.

3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4) ☒ Claim(s) 38-67 is/are pending in the application.

4a) Of the above claim(s) 40,41,55,56,60-62,65 and 67 is/are withdrawn from consideration.

5) ☐ Claim(s) _____ is/are allowed.

6) ☒ Claim(s) 38,39,42-54,57-59,63,64,66 is/are rejected.

7) ☐ Claim(s) _____ is/are objected to.

8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9) ☐ The specification is objected to by the Examiner.

10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. §§ 119 and 120

12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
 a) ☐ All b) ☐ Some * c) ☐ None of:
 1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
 * See the attached detailed Office action for a list of the certified copies not received.

13) ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application) since a specific reference was included in the first sentence of the specification or in an Application Data Sheet. 37 CFR 1.78.
 a) ☐ The translation of the foreign language provisional application has been received.

14) ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121 since a specific reference was included in the first sentence of the specification or in an Application Data Sheet. 37 CFR 1.78.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)	4) ☐ Interview Summary (PTO-413) Paper No(s). _____
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)	5) ☐ Notice of Informal Patent Application (PTO-152)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____	6) ☐ Other: _____

DETAILED ACTION

Election/Restrictions

The election/restriction of record is maintained.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claim Rejections - 35 USC § 112

Claims 43, 44, 63, 64, 66 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

There is still no support in the specification for the ranges of sperm cells claimed. Applicant ^{has} ~~has~~ not addressed this rejection but it is still valid.

The time limitation still has no support. In fact, as applicant noted, they have support for 1-18 hours, which clearly is more than 6 hours.

Claims 38, 39, 42-54, 57-59, 63, 64, 66 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 63 and 64 are still confusing. The way the applicant has now amended the claims is confusing since it is not clear how the "at least some of said sex selected sperm cells" can be not frozen as in claims 63 and 64 and indeed frozen in the main claim, claim 38. If claim 38 requires that the "at least some of said sex selected sperms cells" are to be frozen then claims 63 and claim 64 fail to further limit claim 38.

Claims 38, 39, 42-54, 57-59, 63, 64, 66, are rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. in view of Spaulding and Shrimpton.

Applicant argues that the main claim now has flow cytometry in it. Applicant states that one would not want to use flow cytometry since it is argued that flow cytometry would not preserve sperm viability. This is not well taken since this rejection is a combination of references and flow cytometry is well known in the art as is evident from the cited references. The reference clearly sex selected sperm cells using flow cytometry, thus the claimed invention is known.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 703-308-4230. The examiner can normally be reached on Monday thru Friday: 9:00am-5:30pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brenda Brumback can be reached on 703-306-3220. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-0196.



Michael V. Meller
Primary Examiner
Art Unit 1654

MVM

Index of Claims



Application No.

09/478,299

Examiner

Michael V. Meller

Applicant(s)

SCHENK, JOHN L.

Art Unit

1654

✓	Rejected
=	Allowed

—	(Through numeral) Cancelled
÷	Restricted

N	Non-Elected
I	Interference

A	Appeal
O	Objected

Claim	Date
Final	Original
1	✓
2	✓
3	✓
4	✓
5	✓
6	✓
7	✓
8	✓
9	✓
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Claim	Date
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149	✓
150	✓

Michael V. Meller

1654

[illegible]

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

Express Mail No: EV 419716875 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

 Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller

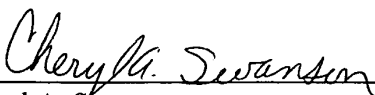
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.
2. I have this day deposited the attached a Request for Continued Examination under 37 C.F.R. §1.114, (RCE) Transmittal form PTO/SB/30 with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 14 day of June, 2004.



Cheryl A. Swanson

06-16-04

Express Mail No: EV 419716875 US

PTO/SB/30 (09-03)

Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Request for Continued Examination (RCE) Transmittal Address to: Mail Stop RCE Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Application Number	09/478,299
	Filing Date	January 5, 2000
	First Named Inventor	John L. Schenk
	Art Unit	1654
	Examiner Name	M. Meller
	Attorney Docket Number	22091-701

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
 Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. **Submission required under 37 CFR 1.114** Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

- a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.
- i. ☐ Consider the arguments in the Appeal Brief or Rely Brief previously filed on _____
- ii. ☐ Other _____
- b. ☒ Enclosed
- i. ☒ Amendment/Reply
- ii. ☐ Affidavit(s)/ Declaration(s)
- iii. ☒ Information Disclosure Statement (IDS)
- iv. ☐ Other _____

2. **Miscellaneous**

- a. ☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
- b. ☐ Other _____

3. **Fees**

- The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
- The Director is hereby authorized to charge the following fees, or credit any overpayments, to
- a. ☐ Deposit Account No. _____
- i. ☐ RCE fee required under 37 CFR 1.17(e)
- ii. ☐ Extension of time fee (37 CFR 1.136 and 1.17)
- iii. ☐ Other _____
- b. ☒ Check in the amount of \$ 1,720.00 enclosed
- c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Name (Print/Type)	Luke Santangelo	Registration No. (Attorney/Agent)	31,997
Signature	<i>Luke Santangelo</i>	Date	12 June 2004

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as express mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.			
Name (Print/Type)	Cheryl A. Swanson	Date	6/17/2004
Signature	<i>Cheryl A. Swanson</i>		

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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Express Mail No: EV 419716875 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller

Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

AMENDMENT AND REQUEST FOR RECONSIDERATION
UNDER 37 C.F.R. § 1.114

I. INTRODUCTORY REMARKS

The Applicant hereby submits the following amendments and explanation in this case pursuant to 37 C.F.R. 1.114, as a Request for Continued Examination. This is filed in response to the action dated December 24, 2003. To respond to all issues, this amendment is submitted in accordance with the amendment directions provided in 37 CFR 1.121 as now existing. Each amendment is believed to have been made in accordance with Rule 121, however, should any unintended informality exist, it is requested that the undersigned be contacted by telephone so that it may be resolved as expediently as possible.

This Request for Continued Examination is appropriate because:

- i) this request is made prior to payment of the issue fee [37 CFR 1.114 (a)(1)];
- ii) the application has not been abandoned;
- iii) no notice of appeal has been filed [37 CFR 1.114 (a)(3)];
- iv) prosecution on the application is closed by either a final action or issuance of a notice of allowance [37 CFR 1.114 (b)];

- v) this application is not a provisional application [37 CFR 1.114 (e)(1)];
- vi) this is a not utility patent application filed before June 8, 1995 [37 CFR 1.114 (e)(2)];
- vii) this application is not an international application filed under 35 U.S.C. 363 before June 8, 1995 [37 CFR 1.114 (e)(3)];
- viii) this application is not a design patent application [37 CFR 1.114 (e)(4)]; and
- ix) this is not a patent under reexamination [37 CFR 1.114 (e)(5)].

Amendments to the Claims begin on page 3 of this paper.

Remarks begin on page 9 of this paper.

II. AMENDMENTS TO THE CLAIMS:

Listing of Claims:

The following listing of claims should be entered to replace all prior listings of claims in the application. In accordance with Rule 121, the status of each claim is indicated parenthetically. In this regard, please note that the brackets in claims 49 and 60 are intended to be part of the claim and do not indicate a deletion. As can be seen in this listing, claims 38 and 67 have been amended, claim 66 has been cancelled and claims 39, 45-54, and 57-59 remain unchanged. Claims 38, 42-54, 57-59, 63-64, and 67 remain in the application.

Claims 1-37 (Cancelled)

38. (Currently amended)

A method of freezing sex-selected sperm cells, comprising:

- (a) obtaining sperm cells of a species of mammal;
- (b) sorting said sperm cells, without the presence of protective compounds in seminal plasma, and based upon sex-type ~~using flow cytometry~~ to create a collection of sex-selected sperm cells;
- (c) cooling said sex-selected sperm cells;
- (d) isolating at least some of said sex-selected sperm cells to create a collection of isolated sex-selected sperm cells;
- (e) suspending at least some of said isolated sex-selected sperm cells in an extender to at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender;
- (f) freezing said isolated sex-selected sperm cells in said extender; and
- (g) thawing said isolated sex-selected sperm cells to provide fertile sex-selected sperm cells.

39. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 38, wherein said

sperm cells from said species of said male mammal are selected from the group consisting of bovine sperm cells and equine sperm cells.

40. (Withdrawn)

The method of freezing sperm cells as described in claim 39, wherein said step of isolating at least some of said sex-selected sperm cells comprises isolating a number of bovine sperm cells between about 300,000 and about 3,000,000.

41. (Withdrawn)

The method of freezing sperm cells as described in claim 39, wherein said step of isolating at least some of said sex-selected sperm cells comprises isolating a number of bovine sperm cells of no more than about 1,000,000.

42. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal comprise equine sperm cells.

43. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 42, wherein said step of isolating at least some of said sex-selected sperm cells comprises isolating a number of equine sperm cells between about 1,000,000 million and about 25,000,000.

44. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 42, wherein said step of isolating at least some of said sex-selected sperm cells comprises isolating a number of equine sperm cells of no more than about 5,000,000.

45. (Previously presented)

The method of freezing sex-selected sperm cells as described in claim 38, wherein said step of cooling sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells to about 5°Celsius.

46. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 45, wherein said step of reducing the temperature of said sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells over a period of about 60 minutes to about 240 minutes.
47. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 38, wherein said extender further comprises a component which maintains osmolality and buffers pH.
48. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.
49. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris[hydroxymethyl]aminomethane, 200mM Tris[hydroxymethyl]aminomethane, 175 mM to 225mM Tris[hydroxymethyl]aminomethane, 200 mM Tris[hydroxymethyl]aminomethane/65mM citric acid monohydrate, 175 mM to 225mM Tris[hydroxymethyl]aminomethane/50mM to 70mM citric acid monohydrate, N-Tris [hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/65 mM citric acid monohydrate, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/50mM to 70 mM citric acid monohydrate, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

50. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 47, 48, or 49, wherein said extender has a pH in the range of about 6.5 to about 7.5.
51. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 50, wherein said extender further comprises a cold shock protectant.
52. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 51, wherein said cold shock protectant is selected from the group consisting of egg yolk, 20% egg yolk, 15% to 25% egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.
53. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 51, wherein said extender further comprises an energy source.
54. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 53, wherein said energy source is selected from the group consisting of a saccharide, glucose, fructose, 56 mM fructose, 45mM to 60mM fructose, mannose, and any combination thereof.
55. (Withdrawn)
The method of freezing sex-selected sperm cells as described in claim 53, wherein said extender further comprises an antibiotic.

56. (Withdrawn)
The method of freezing sex-selected sperm cells as described in claim 55, wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, lincospectin, spectinomycin, penicillin, streptomycin, and any combination thereof.
57. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 47, 51, 53, or 55, wherein said extender further comprises a cryoprotectant.
58. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.
59. (Previously presented)
The method of freezing sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of glycerol, 6% glycerol, between 5% to 7% glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.
60. (Withdrawn)
The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which at least some of said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.
61. (Withdrawn)
The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which at least some of said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, egg yolk, and one or more antibiotics.

62. (Withdrawn)

The method of freezing sex-selected sperm cells as described in claim 38, wherein the extender in which at least some of said sex-selected sperm cells are suspended comprises glycerol, egg yolk, milk, fructose, and one or more antibiotics.

63. (Previously Presented)

The method of freezing sex-selected sperm cells as described in claim 38, further comprising the step of equilibrating at least some of said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to freezing over a period of about 1 hour to about 18 hours.

64. (Previously Presented)

The method of freezing sex-selected sperm cells as described in claim 59, further comprising the step of equilibrating at least some of said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to freezing over a period of not greater than 6 hours.

65. (Withdrawn)

A frozen sex-selected sperm sample in accordance with the method of claim 38.

66. (Canceled)

67. (Withdrawn-Currently Amended)

The method of freezing sperm cells as described in ~~claim 66~~ claim 38, wherein said step of freezing said sex-selected sperm cells in said extender comprises freezing a number of bovine sperm cells between about 300,000 and about 5,000,000.

III. Remarks

Amendments to the claims have been made to refine claim language as discussed in the October 2003 interview. As to the issues raised by the examiner, it should be understood that support does exist in the specification for the current and previous amendments. Among other locations, attention is directed to pages 11 and 19 for the particular dilution values set forth in the claim. As discussed in the interview of October 29, 2003, these amendments are made to address the inventive aspect focused upon, namely, the dilution effect upon the sperm cell viability and effectiveness. This amendment is believed to address all the questions raised in the prior action, but as discussed in the interview, if the examiner believes additional explanation is necessary, the undersigned requests that a telephone call so that affidavit(s) or further explanation can be provided in the most expeditious manner. Although previously withdrawn, claim 67 is amended. It is requested that this claim be re-instated to the application as now dependent upon independent claim 38.

Finally, for clarification and in light of recent case law, it should be understood that the amendments made herein should not be interpreted as an action that in any way surrenders a particular equivalency, surrenders any patent coverage, surrenders any right to patent coverage, or otherwise limits any rights which the applicant may now or hereafter assert. It should be understood that, unless and to the extent deemed broadened by this amendment, and even as amended, the applicant expressly reserves all rights, including but not limited to: all rights to maintain the scope of literal coverage with respect to any element as may have existed under the language previously presented, all rights to maintain the scope of equivalency coverage, and all rights to re-present the prior language at any time in this or any subsequent application. In both the initial drafting of claims and in the amendments made herein, it should also be understood that the applicant has intended to capture as full and broad a scope of coverage as legally available. To the extent that insubstantial substitutes are made, to the extent that the applicant did not in fact draft any claim so as to literally encompass any particular embodiment, and to the extent otherwise applicable, the applicant should not be understood to have in any way intended to or actually relinquished such coverage as the applicant simply may not have been able to

anticipate all eventualities; one skilled in the art, should not be reasonably expected to have drafted a claim that would have literally encompassed such alternative embodiments.

CONCLUSION

In accordance with the amendment directions provided in 37 CFR 1.121, as recently changed, please amend claims 38 and 67 and cancel claim 66. Each amendment is believed to have been made in accordance with Rule 121, however, any unintended informality exist, it is requested that the undersigned be contacted by telephone so that it may be resolved as expediently as possible. The Applicant respectfully requests that the Examiner reconsider the application and allow all claims at his earliest convenience.

Dated this 12th date of June 2004.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By:



Luke Santangelo
Attorney for Applicant
PTO No. 31, 997
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

Express Mail No: EV 419716875 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller

Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

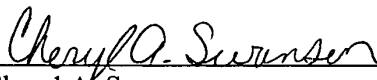
CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day deposited the attached Petition for Extension of Time under 37 C.F.R. § 1.136(a) with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 14 day of June, 2004.



Cheryl A. Swanson



Express Mail No: EV 419716875 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
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Title: Method of Cryopreserving Selected Sperm Cells
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Examiner: M. Meller

Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

PETITION FOR EXTENSION OF TIME UNDER 37 C.F.R. § 1.136(a)

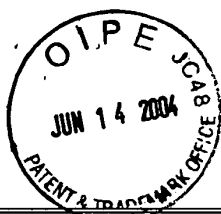
On behalf of the applicant, the undersigned attorney hereby requests an extension of time pursuant to 37 C.F.R. § 1.136(a). This request is submitted in relation to a response due to the office action mailed December 24, 2003, having a shortened statutory period for response of three months. A response to this action on the merits would otherwise be due March 24, 2004; however, it is requested that this time period be extended for three months to and including June 24, 2004. This extension will thereby encompass the statutory period of six months pursuant to 35 U.S.C. §133. Pursuant to 37 C.F.R. §1.17, an extension fee of \$950.00 is submitted with this Petition. In the event the fee has been inappropriately calculated, please allow the maximum extension possible and inform me at your earliest convenience so that any deficiency or error can be corrected immediately.

Dated this 12th day of June, 2004

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By: John Santangelo
Luke Santangelo
Attorney for Applicant
USPTO Reg. No. 31,997
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

06/17/2004 WABDELRI 00000131 09478299 950.00 UP
02 FC:1253



Express Mail No: EV 419716875 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT	DOCKET NO. XY Frozen RCE	Serial No. - 09/478,299
	APPLICANT (S) John L. Schenk	
	FILING DATE January 5, 2000	ART UNIT - 1654

I. U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO	DATE	NAME	CLASS	SUBCLASS	FILING DATE
	6,395,305 B1	05/08/2002	Buhr et al.	424	520	09/22/1998

II. FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						Yes	No

III. OTHER DOCUMENTS

EXAMINER	DATE CONSIDERED
EXAMINER: Please initial if citation considered, whether or not citation is in conformance with MPEP § 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.	



Express Mail No: EV 419716875 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller

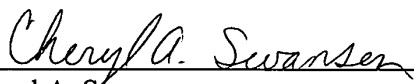
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.
2. I have this day deposited the attached Information Disclosure Statement (Per the OG Notice dated 05 August 2003, no copy of the reference is included as the reference is a US patent) with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 14 day of June, 2004.



Cheryl A. Swanson



Express Mail No: EV 419716875 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
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Attorney Docket: 22091-701
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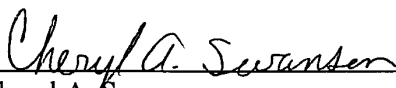
CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day deposited the attached Letter of Transmittal, along with a check in the amount of \$1,720.00 (\$770.00 for the RCE fee and \$950.00 for the 3-month extension of time fee), and a return postcard with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 14 day of June, 2004.


Cheryl A. Swanson



Express Mail No: EV 419716875 US

IN THE UNITED STATES PATENT AND
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Application Number: 09/478,299
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Group Art Unit: 1654
Examiner: M. Meller

Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

LETTER OF TRANSMITTAL

To: Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Office Action mailed December 24, 2004, enclosed for filing are the following documents:

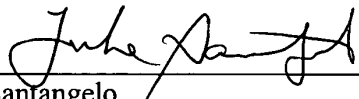
1. a Petition for Extension of Time under 37 C.F.R. §1.36(a);
2. a Request for Continued Examination under 37 C.F.R. §1.114, (RCE) Transmittal form PTO/SB/30;
3. an Amendment and Request for Reconsideration Under 37 C.F.R. § 1.114;
4. an Information Disclosure Statement (Per the OG Notice dated 05 August 2003, no copy of the reference is included as the reference is a US patent);
5. this Letter of Transmittal along with a check in the amount of \$1,720.00 (\$770.00 for the RCE fee and \$950.00 for the 3-month extension of time fee);
6. Certificates of Express Mailing for the above documents and a postcard receipt for return to the undersigned.

Please address all future correspondence to: Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, CO 80521.

Please confirm receipt of the documents by applying your date stamp on the enclosed postcard and returning it to me.

Dated this 12th day of June, 2004

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By: 
Luke Santangelo
Attorney for Applicant
USPTO Reg. No. 31,997
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100



UNITED STATES PATENT AND TRADEMARK OFFICE

42

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509
	7590	08/26/2004		
Craig Miles Santangelo Law Offices P C 125 South Howes Street 3rd Floor Fort Collins, CO 80521			EXAMINER MELLER, MICHAEL V	
			ART UNIT	PAPER NUMBER
			1654	

DATE MAILED: 08/26/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No. 09/478,299		Applicant(s) SCHENK, JOHN L.	
	Examiner Michael V. Meller		Art Unit 1654	
	-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --			

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on 14 June 2004.

2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.

3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4) ☒ Claim(s) 38-65 and 67 is/are pending in the application.

 4a) Of the above claim(s) 40,41,55,56,60-62,65 and 67 is/are withdrawn from consideration.

5) ☐ Claim(s) _____ is/are allowed.

6) ☒ Claim(s) 38, 39, 42-54, 57-59, 63, 64 is/are rejected.

7) ☐ Claim(s) _____ is/are objected to.

8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9) ☐ The specification is objected to by the Examiner.

10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
 a) ☐ All b) ☐ Some * c) ☐ None of:
 1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892) 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948) 3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.	4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____. 5) ☐ Notice of Informal Patent Application (PTO-152) 6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

The election/restriction of record is maintained.

Claims 40, 41, 55, 56, 60-62, 65, 67 are withdrawn from further consideration as being drawn to non-elected subject matter.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claim Rejections - 35 USC § 112

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

There is still no support in the specification for the ranges of sperm cells claimed. Applicant has not addressed this rejection but it is still valid. There is no support for limitations such as, "to at least about 5 million per milliliter to at least about 10 million

Art Unit: 1654

per milliliter of extender", "equine sperm cells between about 1,000,000 million and about 25,000,000".

The time limitation still has no support. In fact, as applicant noted, they have support for 1-18 hours, which clearly is more than 6 hours.

There is no support for "without the presence of protective compounds in seminal plasma". Nowhere can such support be found. Applicant has not stated where the support is in the specification, and the examiner can find no such support.

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 63 and 64 are still confusing. The way the applicant has now amended the claims is confusing since it is not clear how the "at least some of said sex selected sperm cells" can be not frozen as in claims 63 and 64 and indeed frozen in the main claim, claim 38. If claim 38 requires that the "at least some of said sex selected sperms cells" are to be frozen then claims 63 and claim 64 fail to further limit claim 38.

It is not clear what is meant by, "without the presence of protective compounds in seminal plasma". What protective compounds ? What seminal plasma ?

Further, what is meant by , "to at least about 5 million per milliliter to at least about 10 million per milliliter of extender" ? Is this the concentration of the extender

before it is applied to the sperm cells ? Is the 5 million referring to the number of sperm cells. So how much extender is added to each sperm cell ?

Further, the method of claim 38 is confusing because the preamble states that the method is a method of freezing and the end product of the process is thawed out.

It is also confusing how in step d) at least some of said sex-selected sperm cells are isolated to create a collection of sperm cells. In step b) the sperm cells were isolated when they were sorted. What is the difference ? Further, in step e) it is not clear which "said isolated sex-selected sperm cells" are being claimed. Is it the "at least some of said sex-selected" sperm cells from the beginning of step d) or the collection formed in the second part of step d) ? Further, in step c) what is meant by cooled ? Were they hot to begin with ?

Claim 42 has no antecedent basis for "said male mammal".

Claims 63 and 64 are confusing. First there is no antecedent basis for "the step of equilibrating at least some of said sex-selected sperm cells". What step is this ? Second, what temperature is cooler ? Cooler than what ? This is relative and subjective. There is no point of reference for one to compare it to.

Claim Rejections - 35 USC § 103

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. in view of Spaulding and Shrimpton.

Art Unit: 1654

The newly amended claims still are obvious over the cited prior art of record. Applicant's limitation in claim 38 of "sorting said sperm cells, without the presence of protective compounds in seminal plasma" is met since the references never do this. Applicant has provided no arguments concerning this amendment or support from the specification to support such an amendment. The references are clear about their teachings which are of record.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 571-272-0967. The examiner can normally be reached on Monday thru Thursday: 9:30am-6:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Bruce Campell can be reached on 571-272-0974. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Art Unit: 1654

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

A handwritten signature in black ink, appearing to read 'M. Meller', followed by a horizontal line extending to the right.

Michael V. Meller
Primary Examiner
Art Unit 1654

MVM

Search Notes 	Application No. 09/478,299 Examiner Michael V. Meller	Applicant(s) SCHENK, JOHN L. Art Unit 1654

SEARCHED			
Class	Subclass	Date	Examiner
updated		8/24/04	mm

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
updated	8/24/04	mm

Index of Claims



Application No.

09/478,299

Examiner

Michael V. Meller

Applicant(s)

SCHENK, JOHN L.

Art Unit

1654

✓	Rejected
=	Allowed

—	(Through numeral) Cancelled
÷	Restricted

N	Non-Elected
I	Interference

A	Appeal
O	Objected

Claim		Date	
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509

7590 02/22/2005

Craig Miles
Santangelo Law Offices P C
125 South Howes Street
3rd Floor
Fort Collins, CO 80521

EXAMINER

MELLER, MICHAEL V

ART UNIT

PAPER NUMBER

1654

DATE MAILED: 02/22/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Interview Summary	Application No.	Applicant(s)	
	09/478,299	SCHENK, JOHN L.	
	Examiner	Art Unit	
	Michael V. Meller	1654	

All participants (applicant, applicant's representative, PTO personnel):

- (1) Michael V. Meller. (3) _____
 (2) Luke Santangelo. (4) _____

Date of Interview: 17 February 2005.

Type: a) ☐ Telephonic b) ☐ Video Conference
 c) ☒ Personal [copy given to: 1) ☐ applicant 2) ☒ applicant's representative]

Exhibit shown or demonstration conducted: d) ☐ Yes e) ☒ No.
 If Yes, brief description: _____.

Claim(s) discussed: All of record.

Identification of prior art discussed: All of record.

Agreement with respect to the claims f) ☐ was reached. g) ☒ was not reached. h) ☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: Discussed possible amendments to the claims.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.



Examiner's signature, if required

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record

A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews

Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.

All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:

- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

- 1) A brief description of the nature of any exhibit shown or any demonstration conducted,
- 2) an identification of the claims discussed,
- 3) an identification of the specific prior art discussed,
- 4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
- 5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
- 6) a general indication of any other pertinent matters discussed, and
- 7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

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Facsimile No.: 703-872-9306

**IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE**

Application Number:	09/478,299
Applicants:	John L. Schenk
Filed:	January 5, 2000
Title:	Method of Cryopreserving Selected Sperm Cells
Group Art Unit:	1654
Examiner:	M. Meller
Assignee:	XY, Inc.
Attorney Docket:	22091-701
Customer No.:	33549

**RESPONSE AND REQUEST FOR RECONSIDERATION
UNDER 37 C.F.R. § 1.111**

I. INTRODUCTORY REMARKS

This Amendment and Request for Reconsideration is in response to the office communication mailed August 26, 2004 and the personal interview conducted on February 17, 2005. A shortened statutory period of three months has been set, making a response to this action due on or by November 26, 2004. Pursuant to 37 C.F.R. §1.136(a), a petition for extension of time is hereby submitted herewith extending the period for three months to and including February 28, 2005 (February 26, 2005 was a Saturday) along with the appropriate fee. The Applicant submits this amendment and request for reconsideration to fully address the Office's August 26, 2004 action as refined in the personal interview. The Applicant requests that the Office amend the claims as indicated herein.

Amendments to the Claims begin on page 2 of this paper.

Remarks begin on page 8 of this paper.

II. Amendment to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application.

Listing of Claims:

Claims 1-37 (Cancelled)

38. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells, comprising:

- (a) obtaining sperm ~~cells of a~~ cells from a species of a male mammal;
- (b) sorting said sperm cells, without the presence of protective compounds in seminal plasma, and based upon sex-type to create a collection of sex-selected sperm cells;
- (c) cooling said sex-selected sperm cells;
- ~~(d) isolating at least some of said sex-selected sperm cells to create a collection of isolated sex-selected sperm cells;~~
- [[~~(e)~~]] d. suspending ~~at least some of~~ said isolated sex-selected sperm cells in an extender to at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender, and;
- [[~~(f)~~]] e. freezing said ~~isolated~~ sex-selected sperm cells in said extender; and
- ~~(g) thawing said isolated sex-selected sperm cells to provide fertile sex-selected sperm cells.~~

39. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal are selected from the group consisting of bovine sperm cells and equine sperm cells.

40. (Withdrawn/Currently amended)

A method of ~~freezing~~ cryopreserving sperm cells as described in claim 39, and further comprising the ~~wherein said step of isolating at least some of said sex-selected sperm cells comprises isolating~~ a number of bovine sperm cells between about 300,000 and about 3,000,000.

41. (Withdrawn/Currently amended)

A method of ~~freezing~~ cryopreserving sperm cells as described in claim 39, and further comprising the ~~wherein said step of isolating at least some of said sex-selected sperm cells comprises isolating~~ a number of bovine sperm cells of no more than about 1,000,000.

42. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal comprise equine sperm cells.

43. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 42, and further comprising the ~~wherein said step of isolating at least some of said sex-selected sperm cells comprises isolating~~ a number of equine sperm cells between about 1,000,000 million and about 25,000,000.

44. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 42, and further comprising the ~~wherein said step of isolating at least some of said sex-selected sperm cells comprises isolating~~ a number of equine sperm cells of no more than about 5,000,000.

45. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 38, wherein said step of cooling sex-selected sperm cells comprises reducing the temperature of said sex- selected sperm cells to about 5°Celsius.

46. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 45, wherein said step of reducing the temperature of said sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells ~~over a~~ for a period of about 60 minutes to about 240 minutes.

47. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 38, wherein said extender further comprises a component which maintains osmolality and buffers pH.

48. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.

49. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris[hydroxymethyl]aminomethane, 200mM Tris[hydroxymethyl]aminomethane, 175 mM to 225mM Tris[hydroxymethyl]aminomethane, 200 mM Tris[hydroxymethyl]aminomethane/65mM citric acid monohydrate, 175 mM to 225mM Tris[hydroxymethyl]aminomethane/50mM to 70mM citric acid monohydrate, N-Tris [hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]

methyl-2-aminoethanesulfonic acid/65 mM citric acid monohydrate, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/50mM to 70 mM citric acid monohydrate, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

50. (Currently amended)
A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 47, 48, or 49, wherein said extender has a pH in the range of about 6.5 to about 7.5.
51. (Currently amended)
A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 50, wherein said extender further comprises a cold shock protectant.
52. (Currently amended)
A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 51, wherein said cold shock protectant is selected from the group consisting of egg yolk, 20% egg yolk, 15% to 25% egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.
53. (Currently amended)
A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 51, wherein said extender further comprises an energy source.
54. (Currently amended)
A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 53, wherein said energy source is selected from the group consisting of a saccharide, glucose, fructose, 56 mM fructose, 45mM to 60mM fructose, mannose, and any combination thereof.
55. (Withdrawn/Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 53, wherein said extender further comprises an antibiotic.

56. (Withdrawn/Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 55, wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.

57. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 47, 51, 53, or 55, wherein said extender further comprises a cryoprotectant.

58. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.

59. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of glycerol, 6% glycerol, between 5% to 7% glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.

60. (Withdrawn/Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which ~~at least some of~~ said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

61. (Withdrawn/Currently amended) -

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which ~~at least some of~~ said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, egg yolk, and one or more antibiotics.

62. (Withdrawn/Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which ~~at least some of~~ said sex-selected sperm cells are suspended comprises glycerol, egg yolk, milk, fructose, and one or more antibiotics.

63. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 38, further comprising the step of equilibrating ~~at least some of~~ said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to freezing ~~over a~~ for a period of about 1 hour to about 18 hours.

64. (Currently amended)

A method of ~~freezing~~ cryopreserving sex-selected sperm cells as described in claim 59, further comprising the step of equilibrating ~~at least some of~~ said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to freezing over a period of not greater than 6 hours.

65. (Withdrawn)

A frozen sex-selected sperm sample in accordance with the method of claim 38.

66. (Canceled)

67. (Withdrawn/Currently amended)

A method of ~~freezing~~ cryopreserving sperm cells as described in claim 38, wherein said step of freezing said sex-selected sperm cells in said extender comprises freezing a number of bovine sperm cells between about 300,000 and about 5,000,000.

III. Remarks

As agreed in the interview, this response briefly addresses only those aspects of the action that needed clarification consistent with the interview discussion. This included: §112 concerns involving all preambles; an antecedent basis concern; concerns regarding the thawing and isolating steps in claim 38; clarity concerns regarding the "at least some" language; reference to the time limits, protective component, and number values included in certain claims; and finally some discussion regarding §103 questions over the clause regarding the lack of protective compounds. As mentioned in the interview, should any other aspects remain at issue or additional information be desired, it is again requested that for efficiency the examiner contact the undersigned by telephone so that any remaining informalities maybe dealt with as efficiently as possible through a Supplemental Response or Examiner's Amendment as agreed.

As discussed in the interview, the examiner suggested that the claims might be more clear if the preambles were changed from a "method of freezing" to a "method of cryopreserving" and the step of freezing in claim 38 be omitted to be pursued in a divisional application or the like. This suggestion has been following by the above amendments.

Similarly, for clarity reasons the examiner recommended the elimination of the step of "isolating at least some of said sex-selected sperm cells" in claim 38. This has been accomplished as suggested.

In addition, the examiner pointed out that the reference in claim 42 to "said species of said male mammal" lacked proper antecedent basis. This has been corrected through an amendment of claim 38 to reference "a species of a male mammal".

In certain claims, the examiner felt that the commentary to "at least some" of the specified cells or the like was potentially confusing. Such language has been eliminated from claims 38, 40, 41, 43, 44, and 60-64 as suggested.

The examiner also requested highlighting the specification's references discussing the time limits, the protective compounds, and certain number values in the specification.

Claim 63 as amended mentions the aspect of equilibrating for period of about 1 hour to about 18 hours. This aspect is discussed in the specification on page 17 beginning at line 6.

Claim 38 references the aspect of sorting the sperm cells without the presence of the protective compounds in seminal plasma. This aspect and one or more of the specific types of substances effecting protection are mentioned in the specification at page 2, beginning at line 5; at page 7, beginning at line 26; at page 9, beginning at line 1; at page 9, beginning at line 17; at page 16, beginning at line 15; and at page 23, beginning at line 4. Of these, it may be noted that various substances used in extenders and as cryoprotectants are also mentioned.

Claim 38 also references the aspect of 5 million per milliliter of extender to at least about 10 million per milliliter of extender. This aspect and one or more of these specific values are mentioned in the specification at page 19, beginning at line 16; at page 20, beginning at line 11; at page 21, beginning at line 7; at page 22, beginning at line 8; at page 22, beginning at line 18; and at page 23, beginning at line 1. Of particular note and as discussed in the interview, the values and results shown in Table 1 on page 21 and to some degree in Table 2 on page 19 highlight how the claimed ranges show significant in the data. Specifically referencing table 1, it can be seen how the results increase to a more consistent value when the dilution is established at values of at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender.

Claim 43 references the aspect of utilizing between about 1,000,000 million and about 25,000,000 equine sperm. Various dosages and amounts are discussed in the specification. The volume of the straws used for insemination is also mentioned as both 0.25 ml and 0.50 ml at page 17 beginning on line 30 among other locations. Applying the extension mentioned in the specification at page 11 beginning at line 26 and at page 22 beginning at line 8 with these volumes of straws, it can be understood that sperm dosages of 1,250,000 (2.5×10^6 /ml in a 0.50 ml straw) and 25,000,000 (50×10^6 /ml in a 0.50 ml straw) were used. Further, at page 47 beginning at line 17, at page 48 beginning at line 5, at page 51 beginning at line 17, at page 52 beginning at line 25, and also at page 53 beginning at line 4, the use of 1,000,000 (1×10^6) sperm is explicitly mentioned.

Finally, the examiner requested some commentary regarding the aspects of the cryoprotective compounds as not being present in the Spaulding reference. The Spaulding reference proposes the use of antibodies as a vaccine or to achieve sex selection. Besides the fact that this reference has apparently not been shown to actually be achievable -- and for convenience only -- the claims are at this stage distinguished from the Spaulding reference

through their inclusion of the element of sorting the sperm cells without the presence of protective compounds in seminal plasma and suspending at dilutions of at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender. As mentioned in the specification and above, sorting without the presence of protective compounds is a feature that occurs when flow cytometry is used to achieve sex sorting. This technique has been shown to work and is now in practice and is even mentioned in the Spaulding reference (albeit not at the dilutions now set forth). In using this technique, the sperm are stripped of their natural protective compounds and thus are subjected to a more stressful environment. This aspect is not present when using the antibody approach of the Spaulding reference whether it be the immunization of the female (as proposed in Spaulding at column 4, line 43) or for example when binding monoclonal and polyclonal antibodies for sex-enriched semen samples (as proposed in Spaulding at column 4, line 50). Thus, the reference does not address the aspects claims and the section 103 concerns are not believed appropriate.

The amendments submitted herein should be understood to be made as a practicality only, and should not to be construed as creating any situation of file wrapper estoppel or the like as all rights are expressly reserved and may be pursued in this or other applications, such as divisionals, continuations, or continuations-in-part if desired. Relatedly, it should be understood that the amendments made herein are made for tangential issues of clarity and as a matter of the Office's convenience or expedience only. The amendments should not be interpreted as an action that in any way surrenders a particular equivalency, surrenders any right to patent coverage, or otherwise limits any rights which the Applicant may now or hereafter assert. It should be understood that, unless and to the extent deemed broadened by this amendment, and even as amended, the Applicant expressly reserves all rights, including but not limited to: all rights to maintain the scope of literal coverage with respect to any element as may have existed under the language previously presented, all rights to maintain the scope of equivalency coverage as may have existed under the language previously presented, and all rights to re-present the prior language at any time in this or any subsequent application. To the extent currently foreseeable, no change or reduction in direct or equivalency coverage is believed to exist, and no change or reduction in direct or equivalency coverage is intended through the presentation of this amendment.

The Applicant believes all concerns raised in the August 26, 2004 office action have been addressed and that all claims remaining in the case – claims 38, 39, 42-54, 57-59, 63, and 64 – are in condition for allowance. Reconsideration and allowance of these remaining claims is respectfully requested at the Examiner's earliest convenience. Finally, should the Examiner have any remaining questions or disagree with any of Applicant's explanations, it is requested that the Examiner contact the undersigned by telephone in order to expedite the processing of this application as discussed in the interview.

Dated this 28th day of February, 2005.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.


Luke Santangelo
Attorney for Applicant
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FEB-28-2005 MON 08:16 PM

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P. 03

FEB 28 2005

Facsimile No.: 703-872-9306

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller

Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

PETITION FOR EXTENSION OF TIME UNDER 37 C.F.R. §1.136(a)

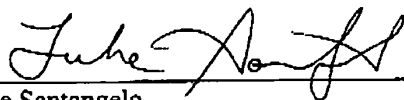
On behalf of the applicant, the undersigned attorney hereby requests an extension of time pursuant to 37 C.F.R. §1.136(a). This request is submitted in relation to a response due to the office action mailed August 26, 2004, having a shortened statutory period for response of three months. A response to this action on the merits would otherwise be due November, 26, 2004; however, it is requested that this time period be extended for three months to and including February 28, 2005 (as February 26, 2005, was a Saturday). This extension will thereby encompass the statutory period of six months pursuant to 35 U.S.C. §133. Pursuant to 37 C.F.R. §1.17, an extension fee of \$1,020.00 is submitted with this Petition. In the event the fee has been inappropriately calculated, please allow the maximum extension possible and inform me at your earliest convenience so that any deficiency or error can be corrected immediately.

Dated this 28th day of February, 2005

Respectfully submitted,

SANTANGELO LAW OFFICES, P.C.

By:



Luke Santangelo
Attorney for Applicant
PTO No. 31,997
125 South Howes, Third Floor
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03/01/2005 EKOL11 00000029 09478299

Resp after RCE-CER

Facsimile No.: 703-872-9306

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller

Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

CERTIFICATE OF FACSIMILE TRANSMISSION

I, Luke Santangelo, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day forwarded the foregoing Petition for Extension of Time under 37 C.F.R. §1.136(a); a Response and Request for Reconsideration Under 37 C.F.R. § 1.111; a Credit Card Payment Form authorized in the amount of \$1,020.00 for the 3-month extension of time fee; a Letter of Facsimile Transmission; and this Certificate of Facsimile Transmission via facsimile number 703-872-9306, Attention Examiner Michael V. Meller, Art Unit 1654.

Dated this 28 day of February, 2005.



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FEB 28 2005

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FACSIMILE TRANSMISSION

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TO: Attention Examiner Michael V. Meller, Art Unit 1654
FAX: 703-872-9306 **TELEPHONE:** 571-272-0967
FROM: Luke Santangelo, USPTO Reg. No. 31,997
DATE: February 28, 2005
TOTAL PAGES: ___ (including this cover page) X Facsimile Filing

LETTER OF FACSIMILE TRANSMITTAL

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

Enclosed for filing in the above application please find the following:

1. a Petition for Extension of Time under 37 C.F.R. §1.136(a) (1 page);
2. a Response and Request for Reconsideration Under 37 C.F.R. § 1.111 (11 pages);
3. a Credit Card Payment Form authorized in the amount of \$1,020.00 for the 3-month extension of time fee (1 page)
4. this Letter of Transmittal (2 pages); and
5. a Certificate of Facsimile Transmittal (1 page).

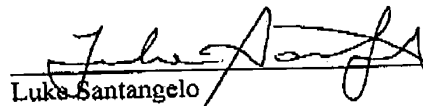
Please confirm receipt of the documents by return facsimile indicating the date of receipt and application number.

I have this 28th day of February, 2005, either myself personally or through my direction of staff at this office, forwarded all of the items in the above Letter of Transmittal via Facsimile Number 703-872-9306, Attention Examiner Michael V. Meller, Art Unit 1654.

Dated this 28th day of February, 2005.

Respectfully Submitted,
SANTANGELO LAW OFFICES, P.C.

By:


Luke Santangelo
Attorney for Applicant
PTO No. 31,997
125 South Howes, Third Floor
Fort Collins, CO 80521
(303) 224-3100



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509
7590 05/13/2005			EXAMINER	
Craig Miles Santangelo Law Offices P C 125 South Howes Street 3rd Floor Fort Collins, CO 80521			MELLER, MICHAEL V	
			ART UNIT	PAPER NUMBER
			1654	

DATE MAILED: 05/13/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No. 09/478,299	Applicant(s) SCHENK, JOHN L.	
	Examiner Michael V. Meller	Art Unit 1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on 28 February 2005.

2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.

3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4) ☒ Claim(s) 38-65 and 67 is/are pending in the application.

4a) Of the above claim(s) 40, 41, 55, 56, 60-62, 65 and 67 is/are withdrawn from consideration.

5) ☐ Claim(s) _____ is/are allowed.

6) ☒ Claim(s) 38, 39, 42-54, 57-59, 63, 64 is/are rejected.

7) ☐ Claim(s) _____ is/are objected to.

8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9) ☐ The specification is objected to by the Examiner.

10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
a) ☐ All b) ☐ Some * c) ☐ None of:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. _____.

3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)	4) ☐ Interview Summary (PTO-413)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)	Paper No(s)/Mail Date. _____
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)	5) ☐ Notice of Informal Patent Application (PTO-152)
Paper No(s)/Mail Date _____	6) ☐ Other: _____

DETAILED ACTION

Election/Restrictions

The election/restriction of record is maintained.

Claims 40, 41, 55, 56, 60-62, 65, 67 are withdrawn from further consideration as being drawn to non-elected subject matter.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claim Rejections - 35 USC § 112

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

There is still no support in the specification for the ranges of sperm cells claimed. Applicant has not addressed this rejection but it is still valid. There is no support for limitations such as, "to at least about 5 million per milliliter to at least about 10 million

per milliliter of extender", "equine sperm cells between about 1,000,000 million and about 25,000,000".

The specification may mention specific points of the concentration (i.e. 1×10^6) but it does not provide for the claimed range of the concentrations (i.e. between about 1,000,000 million and about 25,000,000). The concentrations in table 1 are noted but they are only isolated concentrations, there is no support for ranges of these concentrations, only the specific points tested.

The time limitation still has no support. In fact, as applicant noted, they have support for 1-18 hours, which clearly is more than 6 hours.

Applicant points to the specification at page 17, line 6, but this is not what the problem is. There is no support for "over a period of not more than 6 hours". This is because 1-18 hours clearly is more than 6 hours.

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 63 and 64 are still confusing. The way the applicant has now amended the claims is confusing since it is not clear how the "at least some of said sex selected sperm cells" can be not frozen as in claims 63 and 64 and indeed frozen in the main claim, claim 38. If claim 38 requires that the "at least some of said sex selected sperms cells" are to be frozen then claims 63 and claim 64 fail to further limit claim 38.

It might be clearer if applicant states in claims 63 and 64 that the steps of claims 63 and 64 are performed between steps d) and e) of claim 38.

Further, what is meant by , “to at least about 5 million per milliliter to at least about 10 million per milliliter of extender” ? Is this the concentration of the extender before it is applied to the sperm cells ? Is the 5 million referring to the number of sperm cells. So how much extender is added to each sperm cell ?

This was never addressed by applicant.

Cooling is still not clear in claim 38. Cooling to what temperature ?

Claims 63 and 64 are confusing. First there is no antecedent basis for “the step of equilibrating at least some of said sex-selected sperm cells”. What step is this ? Second, what temperature is cooler ? Cooler than what ? This is relative and subjective. There is no point of reference for one to compare it to.

Claim Rejections - 35 USC § 103

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. in view of Spaulding and Shrimpton.

The claims still are obvious over the cited prior art of record. Applicant’s limitation in claim 38 of “sorting said sperm cells, without the presence of protective compounds in seminal plasma” is met since as applicant notes on page 10 of their latest response filed 2/28/2005, that flow cytometry which is used by Spaulding has been shown to work and is now in practice as applicant has also done. Applicant states on page 10 that sorting

Art Unit: 1654

without the presence of protective compounds is a feature that occurs when flow cytometry is used to achieve sex sorting which Spaulding has also done just like applicant and this is of record. Thus, the references meet the claim limitations.

Applicant also argues that the concentrations of the sperm cells in the extenders is 5 million to 10 million and applicant has pointed to table 1 of the specification to support this, but these points go up and down. For example, the values go up as one approaches 10 million and then decreases as one goes above 10 million. Also, it is not clear what this signifies. It is not clear what significance the increase in the numbers means. Does this increase anything ? Just because one yields higher numbers at 10 million this does not correlate to anything and if it does what does it correlate to ? Further, it is not clear what the 61 refers to in relation to 10 million in table 1. Also it is noted that the values at 24 hours and 48 hours differ in some way. It is not clear what this signifies.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire **THREE MONTHS** from the mailing date of this action. In the event a first reply is filed within **TWO MONTHS** of the mailing date of this final action and the advisory action is not mailed until after the end of the **THREE-MONTH** shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any

Art Unit: 1654

extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 571-272-0967. The examiner can normally be reached on Monday thru Thursday: 9:30am-6:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Bruce Campell can be reached on 571-272-0974. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).



Michael V. Meller

Application/Control Number: 09/478,299

Page 7

Art Unit: 1654

Primary Examiner
Art Unit 1654

MVM

Index of Claims



Application/Control No.

09/478,299

Examiner

Michael V. Meller

Applicant(s)/Patent under Reexamination

SCHENK, JOHN L.

Art Unit

1654

✓	Rejected
=	Allowed

-	(Through numeral) Cancelled
+	Restricted

N	Non-Elected
I	Interference

A	Appeal
O	Objected

Claim	Final	Original	Date
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Michael V. Meller

1654

[illegible]

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

PATENT APPLICATION FEE DETERMINATION RECORD
Effective October 1, 2003

Application or Dockets Number:

09/478299

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)
TOTAL CLAIMS		
FOR	NUMBER FILED	NUMBER EXTRA
TOTAL CHARGEABLE CLAIMS	18 minus 20 =	-
INDEPENDENT CLAIMS	1 minus 3 =	-
MULTIPLE DEPENDENT CLAIM PRESENT ☐		

* If the difference in column 1 is less than zero, enter "0" in column 2

CLAIMS AS AMENDED - PART II

	(Column 1)	(Column 2)	(Column 3)
AMENDMENT A	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	13 Minus	20	-
Independent	1 Minus	3	-
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐			

SMALL ENTITY TYPE ☐ OR

OTHER THAN SMALL ENTITY

RATE	FEE	RATE	FEE
BASIC FEE	340	BASIC FEE	
XS 9=		XS18=	
X43=		X86=	
+145=		+290=	
TOTAL	340	TOTAL	

SMALL ENTITY OR

OTHER THAN SMALL ENTITY

RATE	ADDITIONAL FEE	RATE	ADDITIONAL FEE
XS 9=		XS18=	
X43=		X86=	
+145=		+290=	
TOTAL ADDIT. FEE		TOTAL ADDIT. FEE	

	(Column 1)	(Column 2)	(Column 3)
AMENDMENT B	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	0 Minus	20	=
Independent	0 Minus	3	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐			

RATE	ADDITIONAL FEE	RATE	ADDITIONAL FEE
XS 9=		XS18=	
X43=		X86=	
+145=		+290=	
TOTAL ADDIT. FEE		TOTAL ADDIT. FEE	

	(Column 1)	(Column 2)	(Column 3)
AMENDMENT C	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	0 Minus	20	=
Independent	0 Minus	3	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐			

RATE	ADDITIONAL FEE	RATE	ADDITIONAL FEE
XS 9=		XS18=	
X43=		X86=	
+145=		+290=	
TOTAL ADDIT. FEE		TOTAL ADDIT. FEE	

- * If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
- ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
- *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."
- The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.



Express Mail No: EV 603557196 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

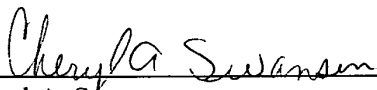
CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day deposited the attached Request for Continued Examination under 37 C.F.R. § 1.114 with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 14th day of November, 2005.



Cheryl A. Swanson



Express Mail No: EV 603557196 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

**REQUEST FOR CONTINUED EXAMINATION
PURSUANT TO 37 C.F.R. § 1.114**

I. INTRODUCTORY REMARKS

The Applicant hereby submits the following submission in this case as a Request for Continued Examination pursuant to 37 C.F.R. § 1.114. This submission consists of an amendment and request for reconsideration and is filed in response to the Office Action dated May 13, 2005. This Request for Continued Examination is appropriate because:

- i) this request is made prior to payment of the issue fee [37 C.F.R. § 1.114 (a)(1)];
- ii) the application has not been abandoned [37 C.F.R. § 1.114 (a)(2)];
- iii) no notice of appeal has been filed [37 C.F.R. § 1.114 (a)(3)];
- iv) prosecution on the application is closed by either a final action or issuance of a notice of allowance [37 C.F.R. § 1.114 (b)];
- v) this application is not a provisional application [37 C.F.R. § 1.114 (e)(1)];
- vi) this is not a utility patent application filed before June 8, 1995 [37 C.F.R. § 1.114 (e)(2)];

- vii) this application is not an international application filed under 35 U.S.C. § 363 before June 8, 1995 [37 C.F.R. § 1.114 (e)(3)];
- viii) this application is not a design patent application [37 C.F.R. § 1.114 (e)(4)]; and
- ix) this is not a patent under reexamination [37 C.F.R. § 1.114 (e)(5)].

Amendments to the Claims begin on page 3 of this paper.

Remarks begin on page 9 of this paper.

This Request for Continued Examination also includes a response to the office communication mailed May 13, 2005. A shortened statutory period of three months has been set, making a response to this action due on or by August 13, 2005. Pursuant to 37 C.F.R. §1.136(a), a petition for extension of time is submitted herewith extending the period for three months to and including November 14, 2005 (as November 13, 2005 was a Sunday) along with the appropriate fee. The Applicant submits this request for reconsideration to fully address the Office's May 13, 2005 action.

II. AMENDMENTS TO THE CLAIMS:

This listing of claims will replace all prior versions, and listings, of claims in the application.

Listing of Claims:

Claims 1-37 (Canceled)

38. (Previously Presented)

A method of cryopreserving sex-selected sperm cells, comprising:

- a. obtaining sperm cells from a species of a male mammal;
- b. sorting said sperm cells, without the presence of protective compounds in seminal plasma, and based upon sex-type to create a collection of sex-selected sperm cells;
- c. cooling said sex-selected sperm cells;
- d. suspending said sex-selected sperm cells in an extender to at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender, and;
- e. freezing said sex-selected sperm cells in said extender.

39. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal are selected from the group consisting of bovine sperm cells and equine sperm cells.

40. (Withdrawn)

A method of cryopreserving sperm cells as described in claim 39, and further comprising the step of isolating a number of bovine sperm cells between about 300,000 and about 3,000,000.

41. (Withdrawn)
A method of cryopreserving sperm cells as described in claim 39, and further comprising the step of isolating a number of bovine sperm cells of no more than about 1,000,000.
42. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal comprise equine sperm cells.
43. (Currently amended)
A method of cryopreserving sex-selected sperm cells as described in claim 42, and further comprising the step of isolating a number of equine sperm cells between about 1,000,000 ~~million~~ and about 25,000,000.
44. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 42, and further comprising the step of isolating a number of equine sperm cells of no more than about 5,000,000.
45. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said step of cooling sex-selected sperm cells comprises reducing the temperature of said sex- selected sperm cells to about 5°Celsius.
46. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 45, wherein said step of reducing the temperature of said sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells for a period of about 60 minutes to about 240 minutes.
47. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein

said extender further comprises a component which maintains osmolality and buffers pH.

48. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.

49. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris[hydroxymethyl]aminomethane, 200mM Tris[hydroxymethyl]aminomethane, 175 mM to 225mM Tris[hydroxymethyl]aminomethane, 200 mM Tris[hydroxymethyl]aminomethane/65mM citric acid monohydrate, 175 mM to 225mM Tris[hydroxymethyl]aminomethane/50mM to 70mM citric acid monohydrate, N-Tris [hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/65 mM citric acid monohydrate, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/50mM to 70 mM citric acid monohydrate, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

50. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, 48, or 49, wherein said extender has a pH in the range of about 6.5 to about 7.5.

51. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 50, wherein said extender further comprises a cold shock protectant.

52. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 51, wherein said cold shock protectant is selected from the group consisting of egg yolk, 20% egg yolk, 15% to 25% egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.
53. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 51, wherein said extender further comprises an energy source.
54. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 53, wherein said energy source is selected from the group consisting of a saccharide, glucose, fructose, 56 mM fructose, 45mM to 60mM fructose, mannose, and any combination thereof.
55. (Withdrawn)
A method of cryopreserving sex-selected sperm cells as described in claim 53, wherein said extender further comprises an antibiotic.
56. (Withdrawn)
A method of cryopreserving sex-selected sperm cells as described in claim 55, wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.
57. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 47, 51, 53, or 55, wherein said extender further comprises a cryoprotectant.

58. (Previously Presented)
- A method of cryopreserving sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.
59. (Previously Presented)
- A method of cryopreserving sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of glycerol, 6% glycerol, between 5% to 7% glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.
60. (Withdrawn)
- A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.
61. (Withdrawn)
- A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, egg yolk, and one or more antibiotics.
62. (Withdrawn)
- A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, egg yolk, milk, fructose, and one or more antibiotics.
63. (Currently amended)
- A method of cryopreserving sex-selected sperm cells as described in claim 38, further comprising ~~the step~~ a step of equilibrating said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to said freezing

step for a period of about 1 hour to about 18 hours.

64. (Currently amended)

A method of cryopreserving sex-selected sperm cells as described in claim 59, further comprising ~~the step~~ a step of equilibrating said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to said freezing step over a period of not greater than 6 hours.

65. (Withdrawn)

A frozen sex-selected sperm sample in accordance with the method of claim 38.

66. (Canceled)

67. (Withdrawn)

A method of cryopreserving sperm cells as described in claim 38, wherein said step of freezing said sex-selected sperm cells in said extender comprises freezing a number of bovine sperm cells between about 300,000 and about 5,000,000.

III. REMARKS

The amendments submitted herein should be understood to be made as a practicality only, and should not to be construed as creating any situation of file wrapper estoppel or the like as all rights are expressly reserved and may be pursued in this or other applications, such as divisionals, continuations, or continuations-in-part if desired. Relatedly, it should be understood that the amendments made herein are made for tangential issues of clarity and as a matter of the Office's convenience or expedience only. The amendments should not be interpreted as an action that in any way surrenders a particular equivalency, surrenders any right to patent coverage, or otherwise limits any rights which the Applicant may now or hereafter assert. It should be understood that, unless and to the extent deemed broadened by this amendment, and even as amended, the Applicant expressly reserves all rights, including but not limited to: all rights to maintain the scope of literal coverage with respect to any element as may have existed under the language previously presented, all rights to maintain the scope of equivalency coverage as may have existed under the language previously presented, and all rights to re-present the prior language at any time in this or any subsequent application. To the extent currently foreseeable, no change or reduction in direct or equivalency coverage is believed to exist, and no change or reduction in direct or equivalency coverage is intended through the presentation of this amendment.

35 U.S.C. § 112 Concerns

The action expressed concerns to the claims under 35 U.S.C. § 112 First and Second Paragraphs. Claim 38 references the aspect of at least 5 million per milliliter of extender to at least about 10 million per milliliter of extender. As previously stated, this aspect and one or more of these specific values are mentioned in the specification at page 19, beginning at line 16; at page 20, beginning at line 11; at page 21, beginning at line 7; at page 22, beginning at line 8; at page 22, beginning at line 18; and at page 23, beginning at line 1. Of particular note and as discussed in the interview on February 17, 2005, the values and results shown in Table 1 on page 21 and to some degree in Table 2 on page 19 highlight how the claimed ranges show significance in the data. Specifically referencing table 1, it can be seen how the results increase to a more

consistent value when the dilution is established at values of at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender. Accordingly, support for the claimed ranges is found in the specification as one skilled in the art can appreciate. The reference to 5 million per milliliter and 10 million per milliliter of extender relates to the concentration of sperm in an extender as discussed in the specification.

Claim 38 also references the aspect of cooling sex-selected sperm cells. Sperm cells can be cooled under suitable conditions within the level of skill in the art. As a non-limiting example, sperm can be cooled to 5 degrees Celsius. Of course, other temperatures may be used as one skilled in the art can appreciate.

The Applicant amends claim 43 to correct a typographical error. Claim 43 references the aspect of utilizing between about 1,000,000 and about 25,000,000 equine sperm. As previously stated, various dosages and amounts are discussed in the specification. The volume of the straws used for insemination is also mentioned as both 0.25 ml and 0.50 ml at page 17 beginning on line 30 among other locations. Applying the extension mentioned in the specification at page 11 beginning at line 26 and at page 22 beginning at line 8 with these volumes of straws, it can be understood that sperm dosages of 1,250,000 (2.5×10^6 /ml in a 0.50 ml straw) and 25,000,000 (50×10^6 /ml in a 0.50 ml straw) were used. Further, at page 47 beginning at line 17, at page 48 beginning at line 5, at page 51 beginning at line 17, at page 52 beginning at line 25, and also at page 53 beginning at line 4, the use of 1,000,000 (1×10^6) sperm is explicitly mentioned. Accordingly, support for the claimed ranges is found in the specification as one skilled in the art can appreciate.

Of note, the language, “at least some” was eliminated from claims 38, 40, 41, 43, 44, and 60-64 in the last response dated February 28, 2005. Accordingly, applicant believes that the examiner’s concerns to the “at least some” language in claims 63 and 64 are moot.

Claims 63 mentions the aspect of equilibrating for period of about 1 hour to about 18 hours. This aspect is discussed in the specification on page 17 beginning at line 6. Further, claim 64 mentions the aspect of equilibrating for a period of not greater than 6 hours. This

aspect is found in the specification on page 17 lines 7-8. Accordingly, these claimed time limits are supported by the specification as one skilled in the art can appreciate. Further, the action suggests that the steps of claims 63 and 64 be clarified as performed between steps d) and e) of claim 38. Applicant believes this is clear since both claims 63 and 64 now include, *inter alia*, “equilibrating ... *prior to said freezing step* ...” (emphasis added).

In addition, claims 63 and 64 mention equilibrating sex-selected sperm cells to a cooler, non-freezing temperature. It may be desirable to cool the temperature of the sperm cells suspended in an extender to any temperature above freezing. As a non-limiting example, sperm can be cooled to 5 degrees Celsius. Of course, other temperatures may be used as one skilled in the art can appreciate.

35 U.S.C. § 103 Concerns

The action expressed concerns to the claims under 35 U.S.C. § 103. A prima facie case of obviousness requires that the combination of references disclose all of the limitations of the claimed invention. As the claims are currently stated, the prior art does not include all of the elements nor limitations of independent claim 38.

To respond to the action’s questions, Table 1 of the specification shows (log) linear relationships for incubation times of 24 hours and 48 hours at different dilutions. Percentages of motile sperm increased as sperm concentration increased to 10×10^6 / ml, (more consistently between 5×10^6 / ml and 10×10^6 / ml) but there was little difference thereafter as seen in Table 1.

As previously noted, the Spaulding reference proposes the use of antibodies as a vaccine or to achieve sex selection. Besides the fact that this reference has apparently not been shown to actually be achievable – and for convenience only -- the claims are at this stage distinguished from the Spaulding reference through their inclusion of the element of sorting the sperm cells without the presence of protective compounds in seminal plasma and suspending at dilutions of at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender. This technique is not mentioned in the Spaulding reference at the dilutions now set

forth. Further, in using this technique, the sperm are stripped of their natural protective compounds and thus are subjected to a more stressful environment. This aspect is not present when using the antibody approach of the Spaulding reference whether it be the immunization of the female (as proposed in Spaulding at column 4, line 43) or for example when binding monoclonal and polyclonal antibodies for sex-enriched semen samples (as proposed in Spaulding at column 4, line 50). Thus, the reference does not address the aspects claims and the section 103 concerns are not believed appropriate.

The Applicant believes all concerns raised in the May 13, 2005 office action have been addressed as best understood and that claims 38, 39, 42-54, 57-59, 63 and 64 remain in the case. Reconsideration and allowance of these remaining claims is respectfully requested at the Examiner's earliest convenience. Finally, should the Examiner have any remaining questions or disagree with any of Applicant's explanations, it is requested that the Examiner contact the undersigned by telephone in order to expedite the processing of this application.

Dated this 14 day of November, 2005.

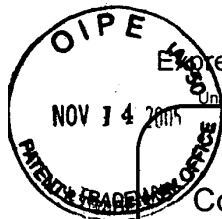
Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.



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11-16-05

IFW/RCE/1654\$



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PTO/SB/30 (04-05)

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Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	09/478,299
Filing Date	01/05/2000
First Named Inventor	John L. Schenk
Art Unit	1654
Examiner Name	Michael V. Meller
Attorney Docket Number	22091-701

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

- Submission required under 37 CFR 1.114** Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).
 - ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.
 - ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
 - ☐ Other _____
 - ☒ Enclosed
 - ☒ Amendment/Reply
 - ☐ Affidavit(s)/ Declaration(s)
 - ☒ Information Disclosure Statement (IDS)
 - ☒ Other Request for Extension of Time;
- Miscellaneous** Letter of Transmittal; Certificates of Express Mailing; Postcard
 - ☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
 - ☐ Other _____
- Fees** The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
 - ☐ The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. _____. I have enclosed a duplicate copy of this sheet.
 - ☒ RCE fee required under 37 CFR 1.17(e)
 - ☒ Extension of time fee (37 CFR 1.136 and 1.17)
 - ☐ Other _____
 - ☐ Check in the amount of \$ _____ enclosed
 - ☒ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	<i>Nicole A. Ressue</i>	Date	November 14, 2005
Name (Print/Type)	Nicole A. Ressue	Registration No.	48,665

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Signature	<i>Cheryl A. Swanson</i>	Date	11/14/05
Name (Print/Type)	Cheryl A. Swanson		

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

790.00 DP

11/17/2005 SFELEKE1 00000033 09478299

01 FC:1601



Express Mail No: EV 603557196 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

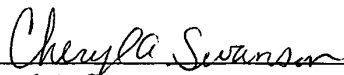
CERTIFICATE OF EXPRESS MAILING

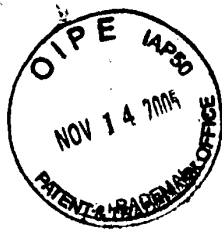
I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day deposited the attached a Request for Continued Examination (RCE) Transmittal with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 14th day of November, 2005.


Cheryl A. Swanson



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Attorney Docket: 22091-701
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PETITION FOR EXTENSION OF TIME UNDER 37 C.F.R. § 1.136(a)

On behalf of the applicant, the undersigned attorney hereby requests an extension of time pursuant to 37 C.F.R. § 1.136(a). This request is submitted in relation to a response due to the office action mailed May 13, 2005, having a shortened statutory period for response of three months. It is requested that this time period be extended for an additional three months to and including November 14, 2005 (November 13, 2005 is a Sunday). This extension will thereby encompass the statutory period of six months pursuant to 35 U.S.C. § 133. Pursuant to 37 C.F.R. § 1.17, an extension fee of \$1,020.00 for the third month of extension is included with this Petition and the accompanying response. In the event the fee has been inappropriately calculated, please allow the maximum extension possible and inform me at your earliest convenience so that any deficiency or error can be corrected immediately.

Dated this 14 day of November, 2005.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By: Nicole A. Ressue
Nicole A. Ressue
Attorney for Assignee
USPTO Reg. No. 48,665
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

11/17/2005 SFELEKE1 00000033 09478299

02 FC:1253

1020.00 OP



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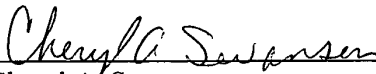
CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day deposited the attached Petition for Extension of Time under 37 C.F.R. § 1.136(a) with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 14th day of November, 2005.


Cheryl A. Swanson



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Application Number: 09/478,299
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Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

LETTER OF TRANSMITTAL

To: Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Enclosed for filing are the following documents:

1. a Petition for Extension of Time under 37 C.F.R. §1.136(a);
2. a Request for Continued Examination (RCE) Transmittal;
3. a Request for Continued Examination under 37 C.F.R. § 1.114;
4. an Information Disclosure Statement along with copies of Foreign Patent Documents and Other References cited;
5. this Letter of Transmittal along with a Credit Card Payment form authorized in the amount of \$1,810.00 (\$790.00 for the RCE fee and \$1,020.00 for the 3-month extension of time fee);

6. Certificates of Express Mailing for the above documents and a postcard receipt for return to the undersigned.

Please address all future correspondence to: Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, CO 80521.

Please confirm receipt of the documents by applying your date stamp on the enclosed postcard and returning it to me.

I have this 14 day of November, 2005, either myself personally or through my direction of staff at this office, deposited all of the items in the above letter of transmittal with the United States Postal Service as Express Mail, postage prepaid, in an envelope addressed to: Mail Stop RCE, Commissioner of Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 14 day of November, 2005

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By: Nicole A. Ressue
Nicole A. Ressue
Attorney for Assignee
USPTO Reg. No. 48,665
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100



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Cheryl A. Swanson
Cheryl A. Swanson



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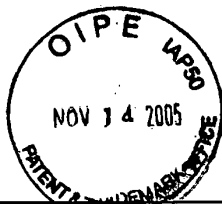
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1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day deposited the attached Information Disclosure Statement along with copies of Foreign Patent Documents and Other References cited with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 14th day of November, 2005.

Cheryl A. Swanson
Cheryl A. Swanson



ExpressMailNo.EV603557196US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICATIONNO:	09/478,299
	FILINGDATE:	1/5/2000
	FIRSTNAMEDINVENTOR	John L. Schenk
	ARTUNIT:	1654
	EXAMINERNAME:	M. Meller
	DOCKETNO:	22091-701

I. US PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO. & KIND CODE (if known)	PATENTEE OR APPLICANT	PUBLICATION/ISSUE DATE	Pages, Columns, Lines Where Relevant Passages Or Relevant Drawings Appear
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Examiner	Date Considered
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01-23-06

RCE/1654
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IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

**FIRST SUPPLEMENT TO
REQUEST FOR CONTINUED EXAMINATION**

This submission is a First Supplement to the Request for Continued Examination filed in the above-referenced case on November 14, 2005. The Request for Continued Examination was filed pursuant to 37 C.F.R. § 1.114 in response to an Office Action dated May 13, 2005, and was filed with an appropriate petition for extension of time.

This First Supplement is submitted to provide the affidavit of John L. Schenk and supporting documents as attached and labeled as Exhibits A - F as well as a supplemental information disclosure statement.

Dated this 20 day of January, 2006.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

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Suppl-resp-text



Express Mail No: EV666038705US

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Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

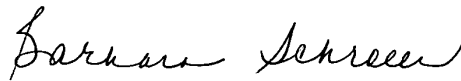
CERTIFICATE OF EXPRESS MAILING

I, Barbara Schroeer, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day deposited the attached First Supplement to Request for Continued Examination with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 20 day of January, 2006.



Barbara Schroeer



Express Mail No: EV666038705US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

LETTER OF TRANSMITTAL

To: Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Enclosed for filing are the following documents:

1. a First Supplement to Request for Continued Examination;
2. an Affidavit under 37 C.F.R. §1.132 including Exhibits A – F;
3. Information Disclosure Statement Pursuant to 37 CFR §1.97(b)(3) and a copy of each Foreign and Other reference cited;
4. this Letter of Transmittal;
5. Certificates of Express Mailing for the above documents and a postcard receipt for return to the undersigned.

Please address all future correspondence to: Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, CO 80521.

Please confirm receipt of the documents by applying your date stamp on the enclosed postcard and returning it to me.

I have this 20 day of January, 2006, either myself personally or through my direction of staff at this office, deposited all of the items in the above letter of transmittal with the United States Postal Service as Express Mail, postage prepaid, in an envelope addressed to: Mail Stop Amendment, Commissioner of Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 20 day of January, 2006.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By: Nicole A. Ressue
Nicole A. Ressue
Attorney for Assignee
USPTO Reg. No. 48,665
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

Suppl-resp-xmt



Express Mail No: EV666038705US

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Barbara Schroeer



IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Confirmation No.: 1509
TC/A.U.: 1654
Examiner: Michael V. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

AFFIDAVIT UNDER 37 C.F.R. § 1.132

1. I, John L. Schenk, a resident of Fort Collins, in the County of Larimer, and State of Colorado, duly sworn and under oath, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

2. Since approximately 1981, I have been involved to varying degrees in the field of sexing spermatozoa and producing animals from such spermatozoa. At the least, this has included work with the Texas A&M University; Colorado State University; Ankony Shadow Isle; and ABS Global, Inc. In fact, I am currently employed by XY, Inc., the assignee of the above referenced patent application. I am also a co-inventor on several patents or patent applications in the field of sexing spermatozoa and producing animals from such spermatozoa, including at least: US Application 60/400,486 entitled "Sperm Cell Process Systems"; US Application 60/400,971 entitled "Low Pressure Sperm Separation System Using Heterospermic Insemination To Assess Sperm Function"; US Application 09/001,394, entitled "Sheath Fluids and Collection Systems for Sex-Specific Cytometer Sorting of Sperm"; US Application 09/015,454, entitled "System for Improving Yield of Sexed Embryo in Mammals"; US Application 09/448,643, entitled "Multiple Sexed Embryo Production System for Mammals"; US Application 09/478,299, entitled "Method of Cryopreserving Selected Sperm Cells"; US Application 09/582,809, entitled "Sex Specific Insemination of Mammals With Low Number of Sperm Cells"; US Application 10/081,955 entitled "Multiple Sexed Embryo Production System for Mammals Using Low Numbers of Spermatozoa"; International Application PCT/US03/24,460 entitled "Low Pressure Sperm Cell Separation System"; and International Application PCT/US00/30,146 entitled "Methods for Improving Sheath Fluids and Collection Systems for Sex-Specific Cytometer Sorting of Sperm". My duties as Reproductive Physiologist for XY, Inc. allow me to review various publications and efforts by others relevant to the field of sexing spermatozoa and producing animals from

BEST AVAILABLE COPY

such spermatozoa and require that I have a certain level of technical expertise in these fields.

3. By virtue of my experience and expertise in the technology of the present invention, I have knowledge and skill which is at least representative of those skilled in the art of cryopreservation of sexed spermatozoa as involved in the above referenced patent application.

4. I have reviewed the following documents:

- a. the specification of U.S. Patent Application No. 09/478,299 as filed on January 5, 2000 ("the Original Application"), attached as Exhibit A;
- b. the claims as filed on November 14, 2005 ("Subject Claims"), attached as Exhibit B;
- c. the examiner's comments ("Subject Examination Comments"), attached as Exhibit C;
- d. the article of Salisbury et al., "Physiology of Reproduction and Artificial Insemination of Cattle" 2nd Ed. San Francisco: W.H. Freeman 442-554 (1978) ("the Salisbury reference"), attached as Exhibit D;
- e. U.S. Patent No. 5,021,244 to Spaulding ("the Spaulding reference"), attached as Exhibit E; and
- f. U.S. Patent No. 4,474,875 to Shrimpton ("the Shrimpton reference"), attached as Exhibit F.

5. I understand that the Subject Claims are to be reviewed by the examiner, and I understand that the bracketed or strikethrough items have been deleted and that the underlined portions are additions to the claims. Further, the Subject Examination Comments have been explained to me as having been provided by the U.S. Patent and Trademark Office, dated May 13, 2005 with respect to the above identified application.

6. Based upon knowledge and skill which is representative of those having ordinary skill in the relevant art, and after review of the Original Application, I am of the opinion that the invention as set forth in the Subject Claims is discussed and supported by the Original Application so as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Further, the Original Application and Subject Claims particularly points out and distinctly claims the subject matter of this invention. I am also of the opinion that the invention as set forth in the Subject Claims is differentiated from the Salisbury reference, the Spaulding reference, and the Shrimpton reference. The basis for these conclusions are listed below.

7. Claim 38 references the range of at least 5 million per milliliter of extender to at least about 10 million per milliliter of extender. This relates to the concentration of sperm in an extender as discussed in the specification. The values and results shown in Table 1 on page 21 and to some degree in Table 2 on page 19 highlight how the claimed ranges show significance in the data. In Table 1, it can be seen how the results increase to a more consistent value when the dilution is established at values of at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender. Specifically, Table 1 shows results of the sperm motility (%) for different sperm concentrations after cooling to 5°C for either 24 hours or 48 hours. As the sperm concentration increases from $1.25 \times 10^6/\text{ml}$ to $5 \times 10^6/\text{ml}$, the sperm motility increases. For example, the sperm motility at $10 \times 10^6/\text{ml}$ is 61% for the 24 hour incubation and 42% for the 48 hour incubation. From these results, I understand that the sperm motility may level off at $5 \times 10^6/\text{ml}$, $10 \times 10^6/\text{ml}$, $15 \times 10^6/\text{ml}$, and perhaps even $20 \times 10^6/\text{ml}$ dilutions. Thus, the results in Table 1 provide dilutions (e.g., at least $5 \times 10^6/\text{ml}$ to at least $10 \times 10^6/\text{ml}$) that may be an appropriate basis for the minimal amount of sperm cells in extenders. While of course other base dilution values may be used, it is clear that there is support in the specification, such as shown in Table 1, for the ranges of “at least about 5 million per milliliter to at least about 10 million per milliliter of extender” as presented in claim 38. Accordingly, I conclude that support for the claimed ranges is found in the Original Application.

8. Claim 43 references the range of between about 1,000,000 to about 25,000,000 equine sperm cells. Various dosages and amounts of sperm cells are discussed in the Original Application (e.g. $50 \times 10^6/\text{ml}$). The volume of the straws used for insemination is also mentioned as both 0.25 ml and 0.50 ml. Applying the extension mentioned in the Original Application with these volumes of straws, it can be understood that sperm dosages of 25,000,000 ($50 \times 10^6/\text{ml}$ in a 0.50 ml straw) were used. Further, the use of 1,000,000 (1×10^6) sperm is explicitly mentioned.

9. As the Original Application notes, extension of sperm samples may produce a suspension of sperm, which may be then transferred into containers for freezing. The cells may be conveniently aliquoted into individual doses sufficient to achieve fertilization. The required dose can vary substantially from one species to the next and is either well-known (e.g. for cattle and horses) or can be readily determined. Therefore, since the Original Application mentions concentrations of sperm of 1,000,000 and 25,000,000 ($50 \times 10^6/\text{ml}$ in a 0.50 ml straw), the Original Application supports the claimed range of the concentrations of between about 1,000,000 to about 25,000,000 based on the substantial variation of doses that may be required. Accordingly, I understand that the claimed range of about 1,000,000 to about 25,000,000 of equine sperm cells is supported by the Original Application.

10. Claims 63 mentions the aspect of equilibrating for period of about 1 hour to about 18 hours. This aspect is clearly discussed in the Original Application. Further, claim 64 mentions the aspect of equilibrating for a period of not greater than 6 hours. The Original Application states, “... sperm are allowed to equilibrate for a period in the range of about

1 hour to about 18 hours, ... preferably between about 3 hours to about 6 hours....” As different times for equilibration may be desired (e.g. 1-18 hours, 3-18 hours, 3-6 hours), claims 63 and 64 covers some of the different time limitations. Further, with respect to claim 64, I understand the text of the Original Application when it states, “to about 6 hours” to describe equilibrating for a period of not greater than 6 hours. Accordingly, these claimed time limits are supported by the specification.

11. In addition, claims 63 and 64 mention equilibrating sex-selected sperm cells to a cooler, non-freezing temperature. A cooler temperature may include cooling sperm cells to a lower temperature. It may be desirable to cool the temperature of the sperm cells suspended in an extender to any temperature above freezing. As a non-limiting example, sperm can be cooled to 5 degrees Celsius. Of course, other temperatures may be used.

12. After reviewing each of the Salisbury reference, Spaulding reference, and Shrimpton reference, I do not find that any of them teach or even suggest all of the elements of independent claim 38. For example, claim 38 is distinguished from these references through the inclusion of the elements of sorting the sperm cells without the presence of protective compounds in seminal plasma and suspending at dilutions of at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender. In addition, I believe that the techniques taught in the Spaulding and Shrimpton references are not capable of being successfully reproduced.

13. Specifically, I have never personally observed a successful accomplishment of the techniques described in the Spaulding reference. I am not aware of any credible evidence suggesting a single instance in which the techniques of the Spaulding reference were successfully reproduced to achieve effective sperm cell discrimination techniques. Further, given the desirability of achieving the results claimed by the Spaulding reference, I believe that the techniques described in the Spaulding reference would be widely practiced if they could be successfully reproduced. Because I have observed that the techniques of the Spaulding reference in fact are not widely practiced, I believe that such techniques cannot be successfully reproduced to increase the probability that offspring will be of the desired sex.

14. Further, I am not aware of any credible evidence beyond that purported to be set forth in the Spaulding reference verifying the successful application to sperm cell discrimination techniques of the use of antibodies to selectively bind to sex-associated membrane proteins. Moreover, the disclosure of the Spaulding reference itself is methodologically insufficient to support a conclusion that the use of antibodies to selectively bind to sex-associated membrane proteins was successfully accomplished as a sperm cell discrimination technique. In particular, the disclosure of the Spaulding reference provides little or no data demonstrating that antibodies were selectively bound to the sex-associated membrane proteins of sperm cells to actually achieve effective separation of such sperm cells into significantly enriched populations of either X-chromosome bearing-sperm cells or Y-chromosome-bearing sperm cells. Examples of such methodological insufficiencies include, but are not limited to, the lack of an explicit statement that an actual enriched population of either X-chromosome-bearing-sperm cells

or Y-chromosome-bearing sperm cells was achieved, the lack of any data regarding what actual purities were achieved for such enriched populations, the lack of any data regarding the methodology used to establish and measure such purities, the lack of any data regarding actual artificial inseminations accomplished with such enriched populations, the lack of any data regarding the methodology used to accomplish such artificial inseminations, and the lack of any data regarding actual sex ratios achieved for the offspring of such artificial inseminations. As a result, I believe that the use of antibodies to selectively bind to sex-associated membrane proteins currently cannot be used to yield populations of sperm enriched for X-chromosome-bearing or Y-chromosome-bearing characteristics nor to increase the probability that offspring will be male or female.

15. Accordingly, it is my opinion that the techniques described in the Spaulding reference are not enabling to accomplish the techniques proposed therein, and therefore could not be successfully accomplished by a person skilled in the art to achieve such techniques.

16. I have never personally observed a successful accomplishment of the techniques described in the Shrimpton reference. Additionally, I am not aware of any credible evidence suggesting a single instance in which the techniques of the Shrimpton reference were successfully reproduced to accomplish the techniques proposed therein. Further, given the desirability of achieving the results claimed by the Shrimpton reference, I believe that the techniques described in the Shrimpton reference would be widely practiced if they could be successfully reproduced. Because I have observed that the techniques of the Shrimpton reference in fact are not widely practiced, I believe that such techniques cannot be successfully reproduced.

17. Moreover, I am not aware of any credible evidence verifying the existence of a difference in the buoyancy of X-chromosome-bearing sperm and Y-chromosome-bearing sperm, and therefore believe that techniques for the identification and discrimination of sperm cells based on such differences in buoyancy cannot be effective. With particular respect to the Shrimpton reference, I believe the evidence of such a difference presented therein is unconvincing. The only evidence of such discrimination between X-chromosome-bearing sperm and Y-chromosome-bearing sperm in the Shrimpton reference is purported to be based on sexes of fetuses from 13 inseminated cows. This evidence is not sufficiently robust to be convincing, especially in the absence of details of experimental design and any evidence of additional replication of these techniques elsewhere in the Shrimpton reference or in other publications. Further, pursuant to my review of the state of the art in this field, I have never observed even a single credible report beyond that purported to be made in the Shrimpton reference of a difference in the buoyancy of X-chromosome-bearing sperm cells and Y-chromosome-bearing sperm cells.

18. Accordingly, it is my opinion that the techniques described in the Shrimpton reference are not enabling to accomplish the techniques proposed therein, and therefore

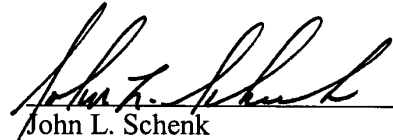
could not be successfully accomplished by a person skilled in the art to achieve such techniques.

19. In conclusion, I disagree with the Subject Examination Comments. First, based upon the Subject Claims, I believe that the Original Application supports and shows possession of the claimed subject matter. Second, based on my review of the references and the Subject Claims, I believe that the references do not teach nor suggest the elements of claim 38. Of course, observations on other aspects may be made, however, it seems unnecessary at present.

20. One of ordinary skill in the art would agree with me in my conclusions stated above.

21. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the application or any patent issued thereon.

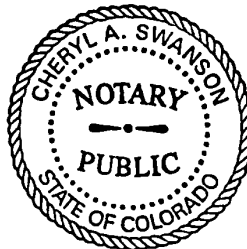
Dated the 20 day of January, 2006.

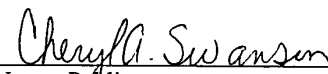

John L. Schenk

UNITED STATES OF AMERICA)
STATE OF COLORADO) ss.
COUNTY OF LARIMER)

SUBSCRIBED AND SWORN to before me in the County of Larimer, State of Colorado, United States of America, by John L. Schenk this 20th day of January, 2006.

WITNESS my hand and official seal pursuant to the authority vested in me as a Notary Public by the State of Colorado.




Notary Public
My Commission Expires: 8/21/2007



Express Mail No: EV666038705US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

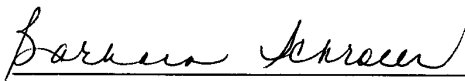
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Applicants: John L. Schenk
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Title: Method of Cryopreserving Selected Sperm Cells
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Dated this 20 day of January, 2006.



Barbara Schroeer

METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 The applications claims the benefit of U.S. Provisional Application No. 60/167,423, filed November 24, 1999.

FIELD OF THE INVENTION

The invention relates to a method for freezing sperm selected for a particular characteristic, as well as to a frozen selected sperm sample and methods of using such a sample.

10 The invention is particularly useful for preserving sex-selected sperm.

BACKGROUND OF THE INVENTION

Over half a century ago, artificial insemination was introduced in the United States as a commercial breeding tool for a variety of mammalian species. Although artificial insemination was initially limited to regions relatively close to the site of sperm collection, advances in the cryopreservation and storage of sperm have facilitated widespread distribution and commercialization of sperm intended for artificial insemination or in vitro fertilization.

15 Further improvements in mammalian sperm collection, selection, cryopreservation, storage, and handling techniques have enhanced the ability of breeders to produce animals having desired traits. For example, advances in selection of mammalian sperm based on slight differences in physical characteristics has made it possible to separate sperm based on sex-type, that is, to select for cells containing either the X or Y chromosome. This technique allows the breeder to manipulate the relative percentage of X- or Y-type sperm in a sample and thereby determine offspring sex. The ability to select sperm based on sex-type or any other desirable characteristic provides an important tool for accelerating genetic progress, increasing production efficiency, and achieving greater flexibility in livestock management. Full exploitation of this tool, however, depends on the ability to freeze and store selected sperm.

20 A variety of methods are available for selecting cells; however, the selection and subsequent processing of sperm presents unique challenges because sperm are incapable of DNA repair and because of sperm morphology. Each sperm has an acrosome overlying the head and a tail, which are important for fertility and which are relatively susceptible to physical injury. In

addition, sperm fertility decreases with increasing time between collection and use. As most of the available selection methods involve physical stresses and take time, selected sperm are typically somewhat compromised compared to non-selected cells. Fertility may be further reduced if the selection technique involves significant dilution. It has been suggested that this

5 "dilution effect" may be due to the loss of protective components in seminal plasma.

Flow cytometry is a particularly efficient selection method that has been employed for sorting sperm by sex-type. However, sorted sperm are subject to stresses beyond those normally encountered in standard artificial insemination or in vitro fertilization protocols. In particular, flow cytometry is time consuming, and, because of the physical constraints of flow cytometers, sperm must be diluted for sorting to levels that are not optimal for storage (usually to on the

10 order of 10^5 - 10^6 /ml). Furthermore, sorted sperm intended for artificial insemination must be concentrated so that conventional packaging and delivery equipment can be used. The need for a concentration step thus exposes already somewhat compromised sperm to additional physical stresses.

15 The freezing of sperm also invariably reduces fertility, motility, and/or viability, and, although techniques for freezing unselected sperm are well known, no technique for cryopreservation of selected sperm has been described.

SUMMARY OF THE INVENTION

20 The present invention provides a method of cryopreserving sperm that have been selected for a specific characteristic. The method is particularly useful for cryopreserving sperm selected by a method that results in dilution of the sperm, since the method provides for the isolation of sperm from a selected sperm sample, followed by addition of a final extender to the isolated sperm to produce a suspension having a desired concentration of sperm. In a preferred

25 embodiment, the method is employed to freeze sex-selected sperm. Although the cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred.

The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample

30 includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or

bison sperm. Also within the scope of the invention is a container including a frozen sperm sample according to the invention.

The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

DETAILED DESCRIPTION OF THE INVENTION

The present invention allows cryopreservation of sperm that have been selected for a particular characteristic, facilitating storage and/or shipment of selected sperm samples to sites distant from the collection site. Thawing yields viable sperm that can be used in procedures such as artificial insemination ("AI") and in vitro fertilization ("IVF"). This result was surprising because of the well-documented fragility of sperm. Prior researchers had demonstrated that the stresses associated with various selection methods or with cryopreservation resulted in significant losses in fertility and/or viability. The present inventors have demonstrated, for the first time, that pregnancies can be achieved with sperm that have been selected and then frozen.

The invention represents an important advance in livestock management, where selection of sperm for use in such procedures can be used to increase the production of offspring having desirable traits. For example, selection to obtain sperm carrying either the X or the Y chromosome allows control over offspring sex, which is advantageous for producers of animals such as dairy or beef cattle. Sex selection also finds application in breeding valuable (e.g., show or race horses) or endangered animals. The ability to freeze selected sperm, which the invention provides, will enable widespread use of such selection methods to, e.g., increase livestock production efficiency as well as quality.

Definitions

The term "acrosome" or "acrosomal cap" refers to the cap that covers the anterior half of the head of sperm and that contains enzymes necessary for ovum penetration.

The term "sex-type" refers to the type of sex chromosome present in the sperm (i.e., the X or Y chromosome).

The term "capacitation" refers to the specific changes a sperm undergoes to develop the capacity to fertilize ova, such as enzymic changes on the surface of the acrosome that lead to release of acrosomal enzymes that facilitate penetration of the sperm into the ovum.

As used with reference to sperm, the term "cryoprotectant" refers to a molecule that protects sperm during a freeze-thaw cycle, promoting survival and retention of fertilizing capacity.

The term "dilution effect" refers to the rapid decline in motility and/or viability of sperm when highly diluted.

As used herein, the term "selection" refers to a method whereby a sample is subdivided based on presence or absence of a specific characteristic (unless context dictates otherwise). Thus, a "selected sperm sample" is a sample obtained by subjecting a source sample to selection for the specific characteristic. A selected sperm sample is therefore enriched, relative to the source sample, in sperm having the specific characteristic.

The term "sorting" is used herein to describe a selection method carried out using a fluorescence-activated cell sorter (FACS).

The term "extender" refers to any medium that tends to preserve sperm viability. The term "extension" refers to the dilution of sperm with extender.

The term "initial extender" refers to a medium used to extend sperm prior to the isolation step of the method of this invention.

The term "final extender" refers to a medium used to extend sperm prior to the freezing step of the method of this invention.

An "organic substance" in an extender described herein is any organic substance that tends to reduce cold shock and preserve fertility of sperm.

An "energy source" in an extender described herein is any substance or substrate that sperm can utilize for cell maintenance and/or motility.

The term "osmolality," as used herein, is a measure of the osmotic pressure of dissolved solute particles in an aqueous solution (e.g., an extender). The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of water.

Cryopreservation Method

The invention provides a method of cryopreserving selected sperm includes the following

steps:

- (1) obtaining a selected sperm sample;
- (2) cooling the selected sperm sample;
- (3) isolating sperm from the selected sperm sample;
- (4) adding final extender to the isolated sperm to produce a suspension of sperm; and
- (5) freezing the suspension of sperm.

10 *Obtaining a Selected Sperm Sample*

The first step in the cryopreservation method of the invention encompasses obtaining a previously selected sperm sample, as well as subjecting a source sample to any suitable selection method. Sperm from any species can be selected and frozen according to the method of the invention. The method can be carried out with sperm from domesticated animals, especially livestock, as well as with sperm from wild animals (e.g., endangered species). Preferably, the selected sperm sample contains mammalian sperm. Human sperm, bovine, equine, porcine, ovine, elk, and bison sperm are particularly preferred.

Generally, the selected sperm sample contains normal, viable sperm. To this end, the ejaculate from which the sperm are obtained typically has at least about 50%, and preferably at least about 75% morphologically normal sperm. In these embodiments, generally at least about 40%, and preferably at least about 60% of the sperm in the ejaculate exhibit progressive motility.

A wide variety of methods for selecting cells from a mixed populations are available, including, for example, selection based on binding of cells or cell components with antibodies, antibody fragments, or other binding partners and differential staining.

The invention is exemplified herein with selection based on sex-type, and sex-selected sperm for use in the invention can be obtained using any selection strategy that takes advantage of slight differences in characteristics between X- and Y-type sperm. Exemplary sex-selection methods include magnetic techniques (see, e.g., U.S. Patent No. 4,276,139), columnar techniques (see, e.g., U.S. Patent No. 5,514,537) gravimetric techniques (see, e.g., U.S. Patent No. 3,894,529, reissue Patent No. 32350, U.S. Patent Nos. 4,092,229, 4,067,965, and 4,155,831). Sex-selection based on differences in electrical properties is disclosed in U.S. Patent

No. 4,083,957, and techniques that select based on differences in electrical and gravimetric properties are discussed in U.S. Patent Nos. 4,225,405, 4,698,142, and 4,749,458. U.S. Patent Nos. 4,009,260 and 4,339,434 describe selection based on differences in motility. Biochemical techniques relying on antibodies are disclosed in U.S. Patent Nos. 4,511,661, 4,999,283, 3,687,803, 4,191,749, 4,448,767, whereas U.S. Patent Nos. 5,021,244, 5,346,990, 5,439,362, and 5,660,997 describe selection based on differences in membrane proteins.

Flow cytometry is a preferred method for separating cells from mixed populations based on differential staining with fluorescent dyes or binding to fluorescently labeled molecules, such as antibodies or nucleic acids. In fluorescence activated cell sorting ("FACS"), cells are "sorted" into different populations based on the fluorescence intensity upon irradiation. FACS can be used for sex-selection of sperm because the X chromosome contains slightly more DNA than the Y chromosome. When sperm are stained with a fluorescent DNA-binding dye, X-chromosome bearing sperm absorb more dye than Y-chromosome bearing sperm and the two populations can therefore be separated by FACS. This strategy was discussed in U.S. Patent No. 4,362,246 and significantly expanded upon in U.S. Patent No. 5,135,759 (issued to Johnson). Separation has been enhanced through the use of high-speed flow cytometers, such as the MoFlo® flow cytometer produced by Cytomation, Inc. (Ft. Collins, CO) and described in U.S. Patent Nos. 5,150,313, 5,602,039, 5,602,349, and 5,643,796, as well as in PCT Publication No. WO 96/12171.

The selection method used to obtain the selected sperm sample is preferably one that preserves sperm viability. Because of the relative fragility of sperm, normal flow cytometry techniques should generally be modified for sorting sperm. More specifically, the flow cytometry entails staining, dilution, and interrogation of cells with light. All of these steps represent stresses that can reduce sperm viability. The sensitivity of sperm to these stresses can vary between species and even between individuals within species. Such sensitivities have either been documented or can readily be determined by empirical studies, such as those described in Examples 1-5.

Modifications that enhance viability are described the patent publications discussed above. For instance, procedures that provide improved sheath and collector systems for sorting sperm are disclosed in PCT Publication No. WO 99/33956 (Application No. PCT/US98/27909). Further, Examples 1-7 below describe exemplary procedures for staining and sorting sperm.

Example 3 describes a study of the effects of laser intensity and dye concentration of post-thaw motility of sorted frozen sperm. This study indicates that the use of lower laser intensities during sorting can increase post-thaw motility.

The selected sperm sample can contain a variety of components besides sperm and will often contain components added to protect the sperm during the selection process. In the case of FACS, the selected sperm sample can contain component(s) of the solutions used for staining and sorting (e.g., the sheath fluid and the catch buffer).

In addition, the selected sperm sample typically contains an extender or extender fraction. For example, "two-step" extenders including an "A fraction" lacking glycerol and a "B fraction" containing glycerol are well known. The A fraction is added to sperm first, followed by addition of an equal volume of the B fraction. For this step, the B fraction is often divided into at least two aliquots and added sequentially; e.g., the second B fraction aliquot is added 15 minutes after the first.

If no extender components are present, an extender or extender fraction is typically added to the selected sperm sample before the sperm are isolated from the sample. If only some extender components are present, additional components can optionally be added so that selected sperm sample includes a complete extender or an extender fraction before the isolation step. In exemplary embodiments, bovine sperm are flow-sorted so as to produce a selected sperm sample including the A fraction of an extender (see Examples 2, 3, and 4). If desired, the B fraction can then be added to the selected sperm sample before the isolation step (see Example 5). The pre-isolation step extender (or extender fraction) is termed "the initial extender" to distinguish it from the "final extender" employed for the extension of isolated sperm before freezing. If the selected sperm sample was selected using FACS, the initial extender can be matched to the sheath fluid employed for sorting. Exemplary matched sheath fluids and extenders are described in detail in Example 4.

An extender suitable for use in the selected sperm sample includes a physiologically acceptable carrier. The physiologically acceptable carrier is typically aqueous, and, in preferred embodiments, includes deionized water. Suitable extenders commonly comprise one or more of the following additional components: a cryoprotectant, a component that maintains osmolality and buffers pH, an organic substance that prevents cold shock and preserves fertility of sperm, a detergent that acts synergistically with certain organic substances to enhance preservation of

sperm, an energy source that can be readily utilized by sperm, an antioxidant, which protects sperm from cold shock, a substance that facilitates sperm capacitation, and one or more antibiotics.

Although cryoprotectants useful in the invention are not limited to those acting by a particular mechanism, most conventional cryoprotectants act, at least in part, by reducing intracellular dehydration. Specifically, freezing is accompanied by an increase in solute concentration in the medium surrounding sperm. This increase in solute concentration draws water out of the cells, which increases intracellular electrolyte concentration. Exemplary cryoprotectants include glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and the like. The cryoprotectant suitable for use in a given extender can vary, depending on the species from which sperm are derived. For example, glycerol is suitable for use in cryopreservation of human and bovine sperm, but is generally not used for cryopreservation of porcine or rabbit sperm. Such preferences are well known for many commercially valuable sperm and can readily be determined empirically for other types of sperm.

The extender useful in the invention optionally includes one or more components that help maintain osmolality and provide buffering capacity. In preferred embodiments of the invention, the osmolality of the extender approximates that of physiological fluids. More preferably, the osmolality of the extender is in the range of about 280 mOsm to about 320 mOsm. The pH is also preferably within a physiologically acceptable range, more preferably in the range of about 6.5 to about 7.5.

Substances helpful in maintaining osmolality and pH within these ranges are well known in the art and can be added to the extender as a solid or already in solution. A buffer containing a salt, a carbohydrate, or a combination thereof can be employed for this purpose. Specific examples include sodium citrate, Tris[hydroxymethyl]aminomethane, and TES (N-Tris [Hydroxymethyl]methyl-2-aminoethanesulfonic acid), and monosodium glutamate buffers; milk; HEPES-buffered medium; and any combination thereof. The component employed to help maintain osmolality and provide buffering capacity in a particular application can vary depending on the other components of the extender and, in some cases, on the species from which the sperm are derived. The selection of such a component for use in the present invention is, however, within the level of skill in the art.

One or more organic substances that protect sperm against cold shock and help preserve fertilizing capacity can also be included in the extender. Such substances are well known and are sometimes described as "nonpenetrating cryoprotectants." One skilled in the art can readily determine an organic substance suitable for a particular application of the cryopreservation method described herein. For example, organic substances containing protective constituents (e.g., lipoproteins, phospholipids, lecithin) that are believed to reduce the impact of cold shock and the dilution effect can be included in the extender. Suitable organic substances include disaccharides, trisaccharides, and any combination thereof. Exemplary organic substances include egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, cholesterol, and any combination thereof.

The extender can also include a detergent. Alkyl ionic detergents, such as sodium dodecyl sulfate (SDS), have been reported to act synergistically with egg yolk to enhance protection against cold shock. Other detergents useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular detergent for a specific application is within the level of skill in the art in light of the guidance provided herein. See, e.g., Example 5.

Preferably, the extender includes an energy source that is readily utilized by sperm. In the absence of an energy source, sperm may oxidize intracellular phospholipids and other cellular components. Thus, the inclusion of an energy source in the extender protects intracellular reserves and cellular components. As is well known in the art, sugars, particularly monosaccharides, provide a convenient energy source, although any conventional energy source can be employed in the extender. Exemplary monosaccharides useful in the extender include glucose, fructose, and/or mannose.

One or more antioxidants can optionally be included in the extender to provide additional protection against cold shock. Exemplary antioxidants include butylated hydroxytoluene (BHT), its derivatives, and the like. However, other antioxidants useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular antioxidant for a specific application is within the level of skill in the art in light of the guidance provided herein.

The extender can also contain a substance that facilitates sperm capacitation. A variety of capacitation facilitators are known in the art and any can be employed in the extender. Examples

include enzymes such as alpha amylase, beta amylase, beta glucuronidase, which can be used in combination, if desired.

Finally, the extender preferably includes an antibiotic, since substantial bacterial growth can threaten sperm viability and increase the risk of infection of the host in artificial insemination or in vitro fertilization procedures. Any of a variety of antibiotics useful in the cryopreservation of cells can also be employed in the extender. The selection of a suitable antibiotic depends on the species from which the sperm was obtained, the procedures involved in obtaining and handling the sperm sample, and the specific microorganism(s) to be targeted. Exemplary antibiotics include tylosin, gentamicin, lincomycin, spectinomycin, linco-spectin (a combination of lincomycin and spectinomycin), penicillin, streptomycin, and ticarcillin, which can be used alone or in combination. However, one skilled in the art can readily determine other antibiotics suitable for use in the extender.

Exemplary extenders are discussed in greater detail below and in the examples.

The sperm concentration is typically lower in the selected sperm sample than in the source sample, and, as indicated above, when FACS is employed, the dilution is significant. A typical sort based on sex-type can produce a sample containing sperm at 6×10^5 cells/ml catch buffer. As such a low concentration is not optimal for storage (at least for most species tested), the cryopreservation method of the invention generally concentrates the selected sperm sample.

Cooling the Selected Sperm Sample

The second step in the cryopreservation method entails cooling the selected sperm sample, typically, by reducing the temperature at a controlled rate. Cooling too rapidly can cause cold shock, which can result in a loss of membrane integrity and cell function. The severity of the effects of cold shock vary from species to species and depend on factors such as the rate of cooling and the temperature range. Under suitable controlled cooling conditions, the sperm are able to adapt to thermal effects. Example 2, among others, describes conditions for cooling bovine sperm, and determining suitable conditions for cooling sperm of other species is within the level of skill in the art.

In a preferred embodiment of the invention, the selected sperm sample is cooled typically from about 22°Celsius, to about 5°Celsius, and cooling is generally carried out over a period of about 60 minutes to about 24 hours, preferably over a period of about 90 minutes to about 240

minutes, and most preferably over a period of about 90 minutes to about 120 minutes. Cooling can be accomplished by any convenient method, including simply placing the selected sperm sample in a 5°Celsius environment.

5 *Isolation of Sperm Cells from the Selected Sperm Sample*

After initial extension of the selected sperm sample, sperm are isolated from the sample using any sufficiently gentle isolation method that provides at least about 50% recovery of sperm, more preferably about 75% to about 90% recovery of sperm, and most preferably about 80% to about 90% recovery of sperm. During the isolation step, the cooled sperm should
10 generally be kept cold, i.e., between about 1 and about 8°Celsius, and preferably close to 4 or 5°Celsius.

Any of a variety of methods suitable for recovering cells from a suspension can be used to isolate the sperm, including for example, filtration, sedimentation, and centrifugation. In an exemplary, preferred embodiment, the selected sperm sample is aliquoted into 50 ml tubes at
15 volumes not exceeding about 27 ml, and preferably between about 20 to about 27 ml. Centrifugation is carried out at about 4°Celsius, at about 850 x g, for about 20 minutes. Preferably, the centrifugation step provides at least about 50% to about 90% recovery of sperm, more preferably about 60% to about 90% recovery of sperm, and most preferably about 70% to about 90% recovery of sperm. After isolation, the supernatant is removed and the pellet is
20 suspended by vortexing gently or repeated aspiration at 4°Celsius. The sperm concentration is then typically determined (e.g., using a hemacytometer).

Final Extension of Isolated Sperm Cells

After isolation, the sperm are pooled, if desired, and extended with final extender to an
25 appropriate concentration for freezing. The concentration of sperm after the final extension and prior to freezing is preferably in the range of about 1×10^6 /ml to about 300×10^6 /ml, more preferably about 10×10^6 /ml to about 50×10^6 /ml, and most preferably about 10×10^6 /ml to about 20×10^6 /ml.

The description of the initial extender above also applies to the final extender, which can
30 be the same as or different from the initial extender. In particular embodiments, the composition



of the sperm sample extended with the final extender is substantially similar to (if not the same as) the composition of the sperm sample after addition of the initial extender.

In a preferred embodiment of the invention, an egg yolk-Tris extender is used. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); citric acid
5 and Tris[hydroxymethyl]aminomethane (buffer); egg yolk (organic substance); fructose (energy source); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

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Components of Egg Yolk-Tris Extender

Glycerol:	4-8% vol/vol
Citric Acid:	55-75 mM
Tris [hydroxymethyl]aminomethane:	190-210 mM
5 Egg yolk:	5-25% vol/vol
Fructose:	45-65 mM
Tylosin:	25-100 µg/ml
Gentamicin:	200-300 µg/ml
Linco-spectin:	100-400 µg/ml*

10 * 100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin

In a variation of this embodiment particularly suitable for freezing bovine sperm, the concentrations of these components after addition of the final extender to the isolated sperm are about 6% (vol/vol) glycerol, about 65 mM citric acid, about 200 mM
15 Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 56 mM fructose, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 150/300 µg/ml linco-spectin (i.e., 150 µg/ml lincomycin and 300 µg/ml spectinomycin), in deionized water.

In an alternative embodiment, an egg yolk-citrate extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); sodium
20 citrate (buffer); egg yolk (organic substance); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

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Components of Egg Yolk-Citrate Extender

Glycerol:	4-8% vol/vol
Sodium Citrate :	60-80 mM
Egg yolk:	5-25% vol/vol
Tylosin:	25-100 µg/ml
Gentamicin:	200-300 µg/ml
Linco-spectin:	100-400 µg/mL*

* 100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin

Exemplary, preferred concentrations for freezing bovine sperm are about 7% (vol/vol) glycerol, about 72 mM sodium citrate, about 20% (vol/vol) egg yolk, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

In another alternative embodiment, an egg yolk-TES-Tris ("EY TEST") extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); egg yolk and heated milk, e.g., homogenized milk containing 1.25% fructose with 10% glycerol (organic substances); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

Components of Egg Yolk TES-Tris Extender

	Glycerol:	3-7% vol/vol
	Tris [hydroxymethy-methyl]-2-aminoethanesulfonic acid:	140-170 mM
5	Tris [hydroxymethyl]aminomethane:	60-80 mM
	Egg yolk:	5-25% vol/vol
	Fructose:	5-12 mM
	Tylosin:	50-150 µg/ml
	Gentamicin:	400-600 µg/ml
10	Linco-spectin:	200-700 µg/mL*
	*200-700 µg/ml lincomycin and 200-700 µg/ml spectinomycin	

Exemplary, preferred concentrations for freezing bovine sperm are about 5% (vol/vol) glycerol, about 158mM Tris[hydroxymethy-methyl]-2-aminoethanesulfonic acid, about 72 mM Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 8mM fructose, about 100 µg/ml tylosin, about 500 µg/ml gentamicin, and about 300/600 µg/ml linco-spectin.

In yet another alternative embodiment of the invention, a Milk extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); heated homogenized milk (organic substance); fructose (energy source); and tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

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Components of Milk Extender

	Homogenized Milk	90% (vol/vol)
	Glycerol:	3-7% (vol/vol)
5	Fructose:	1.25 % (wt/vol)
	Tylosin:	50 µg/ml
	Gentamicin:	250 µg/ml
	Linco-spectin:	250/300 µg/ml

10 * 250-300 µg/ml lincomycin and 250-300 µg/ml spectinomycin

Exemplary preferred concentrations for freezing bovine sperm are about 90% milk, about 10% (vol/vol) glycerol, about 1.25% fructose (wt/vol?), about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

15 Other extenders standardly used to freeze sperm can also be employed as the final extender in freezing selected sperm. A variety of extenders optimized for use in freezing sperm from various species have been described, and many are commercially available. Freezing extenders for equine sperm typically consist of milk, egg yolk, various sugars, electrolytes and a cryoprotectant. Exemplary freezing extenders are described by Squires, E.L., et al., Cooled and
20 Frozen Stallion Semen Animal Reprod. and Biotechnology Laboratory, Bulletin No. 69, Chapter 8, "Seminal Extenders" pp. 49-51 (July, 1999).

Equilibration and Freezing of Sperm

Extension of the sperm sample produces a suspension of sperm, which is then transferred
25 into containers for freezing. If the sperm are intended for use in fertilization, the cells are conveniently aliquoted into individual doses sufficient to achieve fertilization. The required dose can vary substantially from one species to the next and is either well-known (e.g., for cattle and horses) or can readily be determined. In the case of sex-selected bovine sperm, convenient doses range from about 1.0×10^6 sperm to about 3.0×10^6 sperm.

30 Any suitable container can be employed for freezing, including, for example, an ampule, a vial, and a straw. Sperm intended for AI are typically frozen in straws (e.g., 0.25 ml or 0.50 ml

straws) designed for use with an insemination gun. Preferably, a bolus of extender is drawn into the straw, followed, in sequence, by air, sperm, air, and extender, so that the sperm are flanked on either side by an air space, which separates the sperm from a bolus of extender at either end of the straw.

5 Prior to freezing, the sperm are generally allowed to equilibrate at about 5°C. Preferably, the sperm are allowed to equilibrate for a period in the range of about 1 hour to about 18 hours, more preferably between about 3 hours and about 18 hours, and most preferably between about 3 hours and about 6 hours (see Example 2).

10 Following equilibration, any standard freezing method can be employed, provided the freezing rate is not too rapid (i.e., not in excess of about 0.5°C/minute). Preferably, the freezing rate is about 0.5°C/minute. In an exemplary, preferred embodiment, the sperm are placed in static liquid nitrogen vapor, and freezing is carried out in three distinct stages over a period of about 10 minutes. In the first stage of freezing, the sperm are cooled from about 5°C to about
15 -15°C at a rate of about 40°C/minute to about 65°C/minute. In the second stage of freezing, the sperm are cooled from about -15°C to about -60°C at a rate of about 25°C/minute to about 35°C/minute. In the third stage, the sperm are plunged into liquid nitrogen at about -100°C.

Selected Sperm Samples

20 In addition to a freezing method, the invention provides a frozen sperm sample including sperm selected from a source sample for a particular characteristic. The sperm can be from any species, including any of those discussed above with reference to the freezing method. The invention encompasses frozen sperm selected for any characteristic by any suitable method, such as those described above. Preferred embodiments include frozen sex-selected human, bovine, equine, porcine, ovine, elk, or bison sperm. Sex-selection is preferably carried out using flow
25 cytometry as described generally above.

Also within the scope of the invention is a container containing a frozen sperm sample according to the invention. The container can be formed from any material that does not react with the frozen sperm sample and can have any shape or other feature that facilitates use of the sample for the intended application. For samples intended for use in AI, for example, the
30 container is conveniently a straw (e.g., 0.25 ml or 0.5 ml straw) designed for use with an insemination gun. The container is sealed in any manner suitable for preserving the sample at

the intended storage temperature, which is typically below -80°Celsius. 0.25 ml straws can be sealed, for instance, with PVC powder, ultrasonically, or with a cotton-polyvinyl plug and/or a stainless steel ball (BB).

As the frozen sperm sample of the invention is typically thawed before use, the invention
5 also provides a thawed, previously frozen, selected sperm sample and a container including such a thawed sample.

Methods of Using the Selected Sperm Sample

The frozen selected sperm sample of the invention is suitable for use in any method in which sperm are used. The sample can be thawed and used in any conventional fertilization
10 method, such as artificial insemination or in vitro fertilization. Thawing is carried out in the same manner as for frozen, non-selected sperm. Briefly, the straw containing the frozen sperm is submerged in a water bath maintained at a temperature of about 35°C to about 37°C for a period of about 20 to about 30 seconds. After thawing, semen deposition (e.g., insemination) is carried out according to standard procedures, taking care to protect the sperm from environmental
15 fluctuations.

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EXAMPLES

Example 1

Effects Of Dilution On Sperm

- 5 Objective: to determine the effect of sperm concentration on sperm motility for non-frozen, non-sorted, but highly diluted sperm.

A. *Effects of Dilution on Non-washed Sperm*

1. Collection of Source Sample. Sperm were collected from bulls on a routine
10 collection schedule using an artificial vagina as described in Schenk J., Proc 17th NAAB, p.48-58 (1998), and Saacke RG, Proc NAAB Tech Conf AI Reprod. 41:22-27 (1972). All ejaculates used contained greater than 50% progressively motile and greater than 75% morphologically normal sperm. Antibiotics were added to the raw ejaculate as described by Shin S., Proc NAAB Tech Conf AI Reprod. 11:33-38 (1986) within 15 minutes of collection, and the concentration of sperm was determined using a spectrophotometer.

2. Methods. Sperm from 4 bulls were diluted to 1.25, 2.5, 5, 10, 15, and $20 \times 10^6/\text{ml}$ using an egg yolk-citrate extender (EYC) prepared with 20% egg yolk (vol/vol) in 72 mM sodium citrate, 50 $\mu\text{g/ml}$ tylosin, 250 $\mu\text{g/ml}$ gentamicin, and 250/300 $\mu\text{g/ml}$ linco-spectin. Each
20 sample was prepared in duplicate (2 tubes/dilution/bull) and comprised 8 ml total volume per tube. All samples were incubated for 60 minutes at 22°C , after which they were centrifuged using a swinging bucket centrifuge (Eppendorf, Model # 5810R) at $600 \times g$ for 10 minutes to concentrate the sperm. After centrifugation, the supernatant from one set of the duplicate tubes was not removed; the sperm were resuspended in the same medium and at the original
25 concentration by repeated gentle aspiration using a 5-ml serological pipette. (The second set of the duplicate tubes were used in Example 1B.) Sperm samples were then cooled to 5°C at 0.2°C/min over 90 minutes. These sperm were termed "non-washed sperm." All samples were incubated at 5°C for 24 or 48 h post-collection.

3. Evaluation of Motility. After incubation, the samples were warmed to 37°C using
30 a dry block incubator for 10 minutes prior to determination of motility. For this experiment, a

single, blind estimate of the percentage of progressively motile sperm was determined for each sample. Progressive sperm motility was determined subjectively for each subclass by a single observer (x200, phase-contrast microscopy); another person prepared the microscope slides in a randomized manner so the observer was unaware of treatments.

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4. Statistical Analysis. Data were analyzed by analysis of variance (SAS Institute, Cary, North Carolina) with factors bulls and initial dilution concentration. Separate analyses were done for each incubation time. Dilution trends were tested using (log) linear contrasts.

10 5. Results. Data for non-washed sperm (Table 1) revealed (log) linear relationships ($P < 0.01$) for both incubation times. Percentages of motile sperm increased as sperm concentration increased from $1.25 \times 10^6/\text{ml}$ to $10 \times 10^6/\text{ml}$, but there was little difference thereafter. The cubic term was significant ($P < 0.05$) for 24-h and marginally significant ($P < 0.1$) for 48-h incubations. There was a bull effect ($P < 0.01$) at both times.

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Table 1. Effects of cooling on non-washed sperm motility (%) after cooling to 5°C.

Dilution (10 ⁶ /ml)	Incubation at 5°C	
	24 h ^a	48 h ^b
1.25	18 ^c	0 ^c
2.5	38 ^{c,d}	6 ^{c,d}
5.0	56 ^d	31 ^{d,e}
10.0	61 ^d	42 ^c
15.0	55 ^d	44 ^c
20.0	58 ^d	41 ^c
S.E. ^f	5.6	6.4

^a (log) linear (P<0.01) and cubic effects (P<0.05).

^b (log) linear (P<0.01) and cubic effects (P<0.1).

^{c,d,e} Means within columns without common superscripts differ (P<0.05).

^f $\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$
(SAS Institute, Cary, NC, USA)

B. Effects of Dilution on Washed Sperm

1. Collection of Source Sample. The second set of the duplicate tubes containing samples prepared in Example 1A were used in this experiment.

2. Methods. The sperm were diluted, incubated and concentrated by centrifugation as in Example 1A. Following centrifugation, 7.1 ml of the supernatant was aspirated from each tube, removing most of the seminal plasma and leaving the sperm in a 900-μl pellet. The sperm were diluted with EYC (see Example 1A) to make 10 × 10⁶/ml or 20 × 10⁶/ml sperm suspensions. The samples were then cooled to 5°C over 90 minutes as in Example 1A.

3. Evaluation of Motility. The samples were warmed and evaluated for progressive motility as in Example 1A.

4. Statistical Analysis. Data were analyzed as in Example 1A. In addition, data in Example 1B were analyzed for incubation concentration at 5°C.

5. Results. Data for washed sperm (Table 2) revealed no significant treatment effects when sperm were evaluated after 24 h. However, after storage for 48 h at 5°C, there were bull, initial dilution, incubation concentration and bull by incubation effects ($P < 0.05$). More sperm remained motile when held at 20×10^6 /ml than at 10×10^6 /ml (31% vs. 20%; $P < 0.05$). Initial dilutions of 1.25, 2.5, and 5×10^6 sperm/ml resulted in lower progressive motility than 10×10^6 sperm/ml ($P < 0.05$), with respective main effect means of 19, 20, 27, and 37% motile sperm.

Table 2. Cumulative effects of washing, dilution, concentration and cooling on progressive sperm motility (%)

Sperm conc (10^6 /ml) during 1 h preincubation at 37°C	Storage at 5°C – Sperm Concentration and Duration			
	24 h		48 h ^a	
	20×10^6 /ml	10×10^6 /ml	20×10^6 /ml	10×10^6 /ml ^b
1.25	45	49	24	15
2.5	51	40	29	11
5.0	54	54	32	21
10.0	51	50	40	34
15.0	60		41	
20.0	55		40	

^a Concentration to 20×10^6 sperm/ml was superior ($P < 0.05$) to 10×10^6 sperm/ml after 48 h storage.

Also, initial dilution to 10×10^6 was superior to lower dilutions ($P < 0.05$).

Pooled standard errors ($\sqrt{\text{error mean square of ANOVA}} \div \sqrt{N}$) were 4.0 for 24 h, and 2.8 for 48 h incubations.

^b Significant (log) linear trend ($P < 0.06$).

C. Conclusion

High sperm dilution and cooling resulted in a substantial reduction in the percentage of motile sperm, regardless of the presence or removal of seminal plasma. However, this dilution

effect was greatly attenuated by concentrating the diluted sperm to $10 \times 10^6/\text{ml}$ and even more, to $20 \times 10^6/\text{ml}$ before storage at 5°C . Sperm from some bulls tolerated dilution better than sperm from other bulls; however, the bull differences found are typical. Extremely dilute sperm might be compromised during sorting, in part, by removal of protective compounds in seminal plasma.

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Example 2

Effects of Equilibration Time Before Freezing Sorted Sperm

Objective: to evaluate the effect of equilibration times (3, 6 and 18 h, 5°C) before freezing on flow-sorted sperm.

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The following experiment was replicated in its entirety using the same bulls:

1. Collection of Source Sample. Sperm of 4 bulls were collected and prepared as described in Example 1A.

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2. Methods.

a) Staining and Preparation for Sort.

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i) Preparation of Stain Stock Solution: a stock solution of 8.89 mM Hoechst 33342 (bis-Benzimide H-33342; #190305, ICN Biomedicals Inc., Aurora, OH) was prepared in deionized water.

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ii) Sperm Stain Procedure: sperm were diluted in a modified TALP buffer (Table 3) to 400×10^6 sperm/ml. Following dilution, Hoechst 33342 dye was added to the sperm suspensions at a concentration of $224 \mu\text{M}$. After the stain was added to the sperm suspensions, the samples were incubated for 60 minutes at 34°C . Following incubation, sperm were diluted to $100 \times 10^6/\text{ml}$ with TALP containing 2.67% clarified egg yolk and 0.002% food coloring dye (FD&C #40) which quenches the fluorescence of Hoechst 33342 in sperm with compromised cell membranes, allowing them to be gated out during the sorting process. Just prior to flow sorting, samples were filtered at unit gravity through a $40\text{-}\mu\text{m}$ nylon mesh filter to remove any debris and/or clumped sperm.

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b) Sorting. A two-line argon laser operating at 351 and 364 nm and 150 mW was used to excite the Hoechst 33342 dye. The flow cytometer/cell sorter used was an SX MoFlo® (Cytomation, Inc., Fort Collins, CO, USA) operating at 50 psi. A Tris-based sheath fluid was used, consisting of Tris (hydroxymethyl) aminomethane (Tris; 197.0 mM; #T-1503, Sigma Chemical Co., St. Louis, MO, USA), citric acid monohydrate (55.4 mM; #C-7129, Sigma Chemical Co., St. Louis, MO, USA) and fructose (47.5 mM; #F-0127, Sigma Chemical Co., St. Louis, MO, USA). Baseline antibiotics were also added to the Tris-based sheath fluid consisting of 0.58 g/L of penicillin and 0.05 g/L of streptomycin sulfate.

The sperm were sorted by a process referred to as "bulk sorting" which permits rapid accumulation of large numbers of sperm so that large-scale examples can be done within a reasonable time. The sperm pass through the flow cytometer under the standard operating conditions with the exception that all droplets containing viable sperm were collected into a single tube rather than being sorted into 2 tubes based upon specific characteristics (e.g., sorting by sex-type). Sperm were sorted on the basis of viability; hence, sperm that have compromised plasma membranes were excluded during bulk sorting.

Stained sperm were maintained at $22 \pm 1^\circ\text{C}$ during sorting. Bulk sorted sperm were collected in 50-ml plastic tubes containing 2 ml of 20% egg yolk-Tris extender prepared with 20% egg yolk (vol/vol) in 200 mM Tris, 65 mM citric acid, 56 mM fructose, 50 $\mu\text{g/ml}$ tylosin, 250 $\mu\text{g/ml}$ gentamicin, and 150/300 $\mu\text{g/ml}$ linco-spectin in deionized water. The egg yolk-Tris extender was termed "Tris-A fraction" to denote the lack of glycerol at this point in the procedure. Sperm were collected in tubes to contain 12 ml and approximately 6×10^6 sperm. The sperm were subsequently incubated at 22°C for 1 to 3 h to simulate conditions of a sort based on sex-type.

c) Preparation for Freezing. Following incubation, the sorted sperm were cooled to 5°C over the period of 70 minutes. After cooling, the contents of the two tubes were pooled and transferred to a refrigerated, swinging bucket centrifuge set at 5°C and

centrifuged at $850 \times g$ for 20 minutes. After removing the supernatant, processing continued at 5°C by adding about 150 μl of Tris-A fraction extender to about 150- μl of sperm pellet to bring the sperm concentration to approximately $40 \times 10^6/\text{ml}$. The sperm of individual bulls were pooled and diluted immediately with an equal volume of egg yolk-Tris extender containing 12% (v/v) glycerol ("Tris-B fraction"). The Tris-B fraction was added to the sperm suspension as 2 equal volumes at 15-minute intervals to adjust the final sperm concentration to $20 \times 10^6/\text{ml}$. The final glycerol concentration of the complete egg yolk-Tris extender containing the sperm was 6% (v/v).

d) Equilibration and Freezing. Extended sperm were then packaged into 0.25-ml polyvinylchloride straws to be frozen by routine procedures on racks in static liquid nitrogen vapor. Two straws from each of 4 bulls were frozen after 3, 6 and 18 h of total equilibration time at 5°C .

3. Evaluation of Post-Thaw Motility. Straws were thawed in a 37°C water bath for 30 sec. Blind estimates of progressive motility were made after incubating samples at 37°C for 0, 1 and 2 h post-thawing. Each of two observers estimated progressive sperm motility from each of two straws of semen. These four blind estimates for each experimental unit represent subsampling.

4. Statistical Analysis. Statistically, the subsamples were analyzed as a subplot to the main plot least-squares ANOVAs to analyze effects of any observer and observer x treatment interaction. N refers to the number of experimental units, not subsamples; standard errors were calculated on the basis of means of the 4 subsamples from error mean squares of ANOVAs and the numbers of experimental units; least-squares means are presented.

Treatment effects were evaluated via separate ANOVAs for each incubation time. The model included bulls as a random effect and equilibration time and observer as fixed effects; the subplot consisted of the observer term and related interactions.

5. Results. The 3- or 6-h equilibration times were superior to 18-h (Table 4), based on the percentage of progressively motile sperm, for 0 and 1 h ($P<0.01$) but not 2 h of post-thaw incubation. Effects of bull were evident at 1 and 2 h incubation times ($P<0.05$), but not at 0 h. There was no significant ($P>0.1$) bull by equilibration time interaction nor was there a significant observer effect for any response.

Table 3. Modified TALP Buffer

Ingredient	Concentration
NaCl	95.0 mM
KCl	3.0 mM
NaHPO ₄	0.3 mM
NaHCO ₃	10.0 mM
MgCl ₂ • 6H ₂ O	0.4 mM
Na Pyruvate	2.0 mM
Glucose	5.0 mM
Na lactate	25.0 mM
HEPES ^a	40.0 mM
Bovine serum albumin ^b	3.0 mg/ml
Gentamycin Sulfate	30.0 µg/ml

^a #H3375, Sigma Chemical Co., St. Louis, MO, USA

^b #US70195, fraction V; Amersham/Life Science, Cleveland, OH, USA

Table 4. Effect of pre-freeze equilibration time on post-thaw progressive motility (%)

Equilibration at 5°C	Post-thaw incubation at 37°C		
	0 h	1 h	2 h
3 h	41 ^a	36 ^{a,b}	16
6 h	41 ^a	37 ^a	18
18 h	35 ^b	31 ^b	12
S.E. ^c	1.5	0.8	2.0

^{a,b} Within columns, means without common superscripts differ ($P < 0.05$), Tukey's HSD.

^c Pooled standard errors,

$$\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$$

6. Conclusion. The results indicated no differences in post-thaw sperm motility between 3 and 6 h of total equilibration time at 5°C, but there was a significant decline in sperm motility following 18 h of equilibration at 5°C before freezing. The 3- to 6-h range permits pooling 2 consecutive 3-h sorting batches for freezing sperm without decreasing post-thaw motility.

As the bull by equilibration-time interaction was not significant, 3 to 6 h equilibration was adequate, with the caveat that only 4 bulls were used. The optimum equilibration time for a minority of bulls is expected to be >6 h.

Example 3

Effects Of Stain Concentration And Laser Power on Sorted Sperm

Objective: to evaluate the effects of Hoechst 33342 dye concentration in combination with laser intensity on flow-sorted sperm.

5

1. Collection of Source Sample. Sperm of 6 bulls were collected and prepared as described in Example 1A.

2. Methods.

10

a) Experimental Design. One ejaculate (2 bulls) and 2 ejaculates on different days (4 bulls) were used in a 2 by 2 design plus control.

15

b) Staining and Sorting. Staining, preparation for sorting and sorting sperm were achieved as described in Example 2 except that the Hoechst 33342 dye was added to sperm suspensions at a final concentration of 149 μM or 224 μM ; and sperm were bulk-sorted with the laser operating at 100 mW or 150 mW of incident power. Bulk-sorted sperm were collected into 50-ml plastic tubes as described in Example 2. Four tubes containing approximately 15×10^6 total sperm/tube were collected over 1 h for each bull. The sorted sperm were incubated for 1 h at 22°C to simulate a longer sorting time.

20

c) Preparation for Freezing. Following incubation, the sperm were cooled as in Example 2. The sperm were then concentrated by centrifugation at 5°C at $850 \times g$ for 20 minutes. After removing the supernatant, 150 μl of Tris-A fraction extender was added to each 150- μl sperm pellet at 5°C. All of the sperm pellets were suspended by gentle repeated aspiration and the sperm of individual bulls were pooled. Tris B-fraction extender was added stepwise as described in Example 2. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in Tris extender containing 6% glycerol and cooled to 5°C while the bulk-sorted sperm were being prepared.

25

30

d) Equilibration and Freezing. The control and sorted sperm were packaged into 0.25-ml polyvinylchloride straws as described in Example 2, equilibrated at 5°C for 3 h and then frozen conventionally.

5 3. Evaluation of Post-Thaw Motility. Straws were thawed and evaluated as described in Example 2.

10 4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via ANOVA. The model included dye concentration, laser intensity and bulls in the main plot, and observer and related interactions in the subplot. Bulls were considered a random effect and the other factors as fixed.

15 5. Results. Bull effects were significant for percentages of progressively motile sperm immediately after thawing ($P < 0.1$) and after 1 h and 2 h of incubation at 37°C ($P < 0.05$). There was no effect of dye concentration or bull by dye concentration on sperm motility at any incubation time. With bulls considered as a random effect, 150 mW of laser power resulted in lower post-thaw motility of sperm than 100 mW at 0 h of incubation ($P < 0.1$), but not at other incubation times (Table 5). If bulls are considered as fixed effects, 150 mW of power resulted in lower sperm motility than 100 mW ($P < 0.05$) at all 3 incubation times. There was an effect of
20 bull by laser power ($P < 0.05$) on sperm motility at 1 h, but not at 0-h or 2-h incubation times. Also, the higher laser power resulted in lower sperm motility than the control ($P < 0.05$) at 0- and 1-h incubation times (Table 5). There was a significant observer effect at 1-h, but not at 0-h or 2-h, incubation times. There was no observer by treatment interaction ($P > 0.1$).

25

30

Table 5. Effects of laser intensity and dye concentration on post-thaw motility (%).

Main effect means	Incubation at 37°C		
	0 h	1 h	2 h
Control	49	44	33
<u>Dye Concentration</u>			
149 µM	41	39	30
224 µM	42	39	30
<u>Laser Intensity</u>			
100 mW	46	42	33
150 mW	38 ^a	35 ^b	27
S.E. ^c	2.2	1.2	1.3

^a Significant main effect ($P < 0.1$) and differs from control ($P < 0.05$).

^b Differs from control ($P < 0.05$).

^c Pooled standard errors, $\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$

6. Conclusion. Percentages of progressively motile sperm post-thaw were diminished by the staining and sorting process. Higher laser intensity was more damaging than the lower laser intensity. There was no effect of dye concentration on post-thaw sperm motility. Thus, excitation of the sperm-bound Hoechst 33342 dye at lower laser intensities is less damaging and that staining sperm at the higher dye concentration had no detrimental effect on post-thaw motility. The damage observed was presumably to the sperm-motility apparatus.

Example 4

Evaluation of Pre-Sort Staining Procedures and Selection of Extenders for the Cryopreservation Of Sperm

Objective: (1) to evaluate three pre-sort treatments for sperm; and, (2) and to evaluate sheath
5 fluid and extender combinations for the cryopreservation of flow-sorted sperm.

The following experiment was replicated in its entirety:

1. Collection of Source Sample. Sperm from 4 bulls were collected and prepared as
described in Example 1A.

10 2. Methods.

a) Experimental Design. A 3 (pre-sort treatments) by 3 (extenders) by 2
(sheath fluids) by 4 (bulls) by 2 (observers) factorial experiment was designed to
determine the best procedure to hold sperm prior to sorting, and to evaluate three
15 extenders for cryopreserving the sorted sperm. .

b) Sample Preparation and Staining. Freshly collected sperm from each of 4
bulls were treated as follows:

(1) diluted to 400×10^6 /ml in modified TALP (see Example 2, Table 3)
20 and stained for 1 h at 34°C before bulk-sorting ("Dilute - 0 h");

(2) incubated neat at 22°C for 3 h before dilution, staining and sorting
("Neat - 3 h"); or,

(3) diluted and stained as "Dilute-0 h" and then incubated at 22°C for 3 h
25 before bulk-sorting ("Diluted - 3 h").

c) Extenders. The following freezing extenders were compared: EYC (see
Example 1) containing 7% glycerol, egg yolk-Tris (see Example 2) containing 6%
glycerol, and egg yolk-TES-Tris (TEST) containing 5% glycerol. EYC "A
30 Fraction" refers to the EYC extender containing no glycerol, and EYC "B
Fraction" refers to EYC extender containing twice the final, desired glycerol

concentration (i.e., 14%). Thus, when EYC A and B fractions are combined in equal volume, the final EYC extender contains 7% glycerol. Tris A and B fractions are similarly named, and described in Example 2. TEST extender is prepared as a complete extender containing 5% glycerol; hence, there were no "A" and "B" fractions for TEST.

d) Sheath Fluid. Sheath fluid was either 98.6 mM sodium citrate dihydrate (#S279-3, Fisher Scientific, Fair Lawn, NJ) or Tris as described in Example 2. Both types of sheath fluid were adjusted to pH 6.8; osmolality was about 270 to 280 mOsm/kg. Tris sheath fluid was used to collect sperm that were later extended in egg yolk-Tris and TEST freezing extenders. Sheath fluid containing 98.6 mM sodium citrate dihydrate was used to collect sperm to later be extended in EYC freezing extender.

e) Sorting. Approximately 58×10^6 sperm for each combination of pre-sort treatment, sheath fluid and extender were bulk-sorted as described in Example 2 using 150 mW of incident laser power. For each sort, sperm were collected over approximately 1 h. After sorting, the samples were incubated at 22°C for 2 h to simulate a 3 h sort.

f) Preparation for Freezing. Following incubation, the sperm were cooled as described in Example 2. After cooling, the samples were centrifuged at 5°C at $850 \times g$ for 20 min. Each sample comprised about 28 ml total volume and was contained in a 50-ml plastic tube

After the supernatant was removed, the sperm were returned to a 5°C cold room for extension. Samples were extended to 40×10^6 /ml by depositing 131 μ l of the sperm suspension into 69 μ l of A-fraction EYC, A-fraction egg yolk-Tris, or TEST extender. Immediately, suspensions were adjusted to 20×10^6 sperm/ml with the addition of the matched glycerol containing extender (i.e., B-fraction EYC, B-fraction Tris) or TEST. B-fraction extenders were added to their respective samples stepwise (2X) at 15-min intervals as described in Example 2.

The TEST was added to sperm stepwise in the same manner as B-fraction EYC and Tris extenders.

g) Equilibration and Freezing. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for 3 h at 5°C and then frozen in static liquid nitrogen vapor.

3. Evaluation of Post-Thaw Motility. Thawing and post-thaw evaluations of sperm were done as described for Example 2.

4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each post-thaw incubation time. The main plot included pre-sort treatment, extenders, and bulls; the subplot consisted of observers and associated interactions. Bulls were considered a random effect, and the other factors, fixed. The entire experiment was replicated twice. Tukey's HSD test was used to separate means.

5. Results. Post-thaw progressive motility of bulk-sorted sperm was affected ($P < 0.05$) by extender and bulls at each post-thaw incubation time and by pre-sort procedure at 0 h of incubation (Table 6). There were no differences due to sheath fluids ($P > 0.05$). At 0-h post-thaw incubation, use of the neat-3h treatment resulted in more motile sperm after freezing and thawing than the other 2 pre-sort staining treatments ($P < 0.05$; Table 6). However, pre-sort procedures were not statistically significant after post-thaw incubation of sperm for 1 or 2 h with bulls considered as a random effect. Importantly, at these 2 incubation times, there were significant pre-sort treatment by bull interactions ($P < 0.05$). Furthermore, pre-sort treatment would have been a significant effect at all post-thaw incubation times had bulls been considered as fixed effects.

Immediately after thawing (0 h), TEST was the best extender, but after 1 or 2 h of incubation of 37°C, Tris was the best extender. Importantly, there was no pre-sort treatment by extender interaction for any response. There were observer effects ($P < 0.01$) at all incubation

times, but no observer by treatment interactions. There was a bull by extender interaction ($P < 0.05$) at all 3 incubation times.

Table 6. Main effects of pre-sort treatment and freezing extenders on post-thaw progressive motility (%)

Pre-sort procedure	Extender	Incubation at 37°C		
		0 h	1 h	2 h
Dilute - 0 h	Mean	39 ^a	32	22
Neat - 3 h	Mean	43 ^b	36	25
Dilute - 3 h	Mean	38 ^a	31	19
Mean	EYC	36 ^a	29 ^a	17 ^a
Mean	Tris	40 ^b	39 ^b	29 ^b
Mean	TEST	44 ^c	33 ^c	20 ^a
S.E. ^d		0.8	0.8	0.7

^{a,b,c} Means within columns, within main effects, without common superscripts differ ($P < 0.05$).

^d Pooled standard errors = $\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$

6. Conclusion. This study showed that holding sperm neat for 3 h before dilution, staining and sorting was better than immediate dilution and staining 0 h or 3 h later. Thus, by 3 h into the sort, it is best to continue with a new aliquot of the original ejaculate that was held neat 3 h and then stained, rather than continuing with the original sample of sperm stained and held at 400×10^6 sperm/ml.

Even though TEST extender provided higher post-thaw motility at 0 h, Tris was the superior extender when sperm were stressed by incubation at 37°C. Either sheath fluid worked equally well for each extender. Based on these results, we have incorporated the use of Tris sheath fluid in combination with Tris freezing extender into our standard operating procedure.

Example 5

Effects Of Extender Additives on Sorted Sperm

Objective: to evaluate the effect of adding sodium dodecyl sulfate ("SDS") to the freezing extender on flow-sorted sperm.

5

A. *Evaluation of Effect of Concentration of SDS in Freezing Extender*

1. Collection of Source Sample. Sperm of 6 bulls were collected and prepared as described in Example 1A.

10 2. Methods. Sperm from each of 6 bulls were extended to 20×10^6 /ml in 20% whole egg Tris ("WET") extender containing 0, 0.03, 0.06, 0.09, or 0.12 percent SDS, packaged into straws and frozen. WET extender was prepared using 3.028 g of Tris[hydroxymethyl] aminomethane, 1.78 g of citric acid monohydrate, and 1.25 g of fructose per 100 ml of double distilled water, to which 20% whole egg (vol/vol) was added. The WET extender was prepared
15 at a pH of about 7.0 and contained a final glycerol concentration of about 6% (vol/vol). The WET extender also contained 1000 IU of penicillin "G" sodium and 100 µg of streptomycin sulfate/ml.

20 3. Results. The respective means (n = 1 sample from each of 6 bulls) were 51, 51, 50, 51, and 48% progressive motile sperm approximately 10 minutes post-thaw. Based on these results, 0.06 percent SDS was used in Example 5B.

B. *Evaluation of the Effects of 0.06 Percent SDS in Various Freezing Extenders on Post-Thaw Motility of Flow-Sorted Sperm*

25 1. Collection of Source Sample. Sperm of 8 bulls were collected and prepared as described in Example 1A.

30 2. Methods. Post-thaw motility was studied for sperm frozen in egg yolk-Tris (see Example 2) and WET extenders (see Example 5A) with and without 0.06% SDS. Final glycerol content for both extenders was 6%.

a) Staining, Preparation for Sort, Sorting. Stained sperm samples were prepared from an ejaculate from each of 8 bulls as described in Example 2. Stained sperm were bulked-sorted using Tris sheath fluid as described in Example 2 except that the sort was achieved using 135 mW of incident laser power. Sorted sperm were collected in a 50-ml plastic tube containing 2 ml of A-fraction freezing buffer for each extender; 15×10^6 total sorted sperm (25 ml) for each treatment were collected and incubated for 1 h at 22°C to simulate longer sorting.

b) Preparation for Freezing. Diluted sperm were then cooled to 5°C over 90 minutes. An equal volume of appropriate B-fraction extender was added stepwise (2x) at 15-minute intervals to each 50-ml plastic tube containing sorted sperm. Aliquots of 25 ml/extender treatment were concentrated by centrifugation for 20 minutes at 850 x g in a refrigerated centrifuge. The supernatant was removed leaving a 600 µl sperm pellet, which was suspended by gentle vortexing for 15 seconds. No additional extender was added to the sperm pellet since the suspension containing the pellet already contained glycerol. The concentration of the sperm suspension was approximately 20×10^6 /ml. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in egg-yolk-Tris extender containing 6% glycerol. The control was placed in a 5°C cold room while bulk-sorting occurred.

c) Equilibration and Freezing. All control and bulk-sorted sperm were packaged and frozen at the same time. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for about 3 h to about 6 h at 5°C and then frozen in static liquid nitrogen vapor.

3. Evaluation of Post-Thaw Motility. Thawing and post-thaw evaluations of sperm were done as described for Example 2 with the exception that progressive motility was evaluated 0.5 and 2.0 h after incubation.

4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each incubation time; the model included bull and extender in the main plot and observer and

related interactions in the subplot. Differences in means were determined by the least significant difference test.

5. Results. Extender affected ($P < 0.05$) progressive motility of sperm after 0.5 or 2 h post-thaw incubation (Table 7). At 0.5 h, WET plus SDS resulted in lower motility than Tris with SDS. At 2 h, all treatments with bulk-sorted sperm were worse than the non-sorted control sperm. There were significant bull and observer effects ($P < 0.01$) at both incubation times, but no observer by treatment interactions.

Table 7. Effect of extender on post-thaw progressive motility (%)

Extender	Incubation at 37°C	
	0.5 h	2 h
Tris (non-sort)	42 ^a	41 ^a
Tris w/o SDS	40 ^{a,b}	35 ^b
Tris w/SDS	42 ^a	37 ^b
WET w/o SDS	40 ^{a,b}	35 ^b
WET w/SDS	38 ^b	35 ^b
S.E. ^c	1.0	1.2

^{a,b} Means within columns without common superscripts differ ($P < 0.05$).

^c $\sqrt{\text{error mean square of ANOVA} \div \sqrt{N}}$

6. Conclusion. The inclusion of SDS in Tris or WET extenders did not benefit sperm quality as determined by visual estimates of motility after thawing. Also, results using WET and Tris extenders were similar; hence, WET appeared as efficacious as Tris for cryopreserving sorted bovine sperm.

Example 6

Quality of Sperm Sexed by Flow Sorting for Field Trials

Objective: to evaluate post-thaw quality of sorted sperm based on acrosomal integrity.

5 1. Collection of Source Sample. Sperm of 3 bulls were collected and prepared as described in Example 1A.

 2. Methods. Sorted and non-sorted control sperm from the same ejaculate were stained, processed, and sorted as described in Example 2 except the sperm were sorted for sex-
10 type at a 90% purity level. Sorted sperm were collected to a volume of approximately 20 ml and were cooled to 5°C for 90 minutes (0.2°C/min). After cooling, an equal volume of egg yolk-Tris B extender (see Example 2) was added to the sorted sperm in 2 equal volumes at 15-minute intervals. Centrifugation and aspiration of the supernatant were achieved as described in Example 5. After centrifugation and aspiration, egg yolk-Tris extender containing 6% glycerol
15 (v/v) was added to the sperm pellet to bring the concentration of sperm to about 20×10^6 /ml. Freezing and thawing were done as described in Example 2 except that equilibration time was about 3 h.

 3. Evaluation of Post-Thaw Motility. Visual estimates of the percentage of
20 progressively motile sperm at 37°C were made approximately 10 minutes after thawing. The acrosomal integrity of sperm was assessed using differential interference-contrast microscopy (x1000) after 2 h of incubation at 37°C. Sperm were treated with 40 mM sodium fluoride, a wet was smear made, and 100 sperm per treatment were examined. Acrosomes were classified as: (a) intact acrosome, (b) swollen or damaged acrosome, or (c) missing acrosome (non-intact).

25 4. Statistical Analysis. The data analyzed were from 19 different freeze dates balanced across 3 bulls used in field trials. Treatment effects (sort vs. control) were evaluated via analysis of variance with bulls as a fixed effect.

 5. Results. The percentage of progressively motile sperm post-thaw was
30 significantly higher ($P < 0.05$) for non-sorted sperm (50%) than for sorted sperm (46%; Table 8), despite removal of dead sperm during sorting. However, the percentage of sperm with an intact

acrosome was not different. Sorting increased the percentage of sperm missing an acrosome, but also reduced the percentage of sperm with a damaged acrosome, relative to control sperm ($P < 0.05$). There were significant differences among bulls for percent of intact acrosomes ($P < 0.05$), percent of non-intact acrosomes ($P < 0.01$), and post-thaw progressive motility ($P < 0.01$). There was a bull by sorting effect for post-thaw motility ($p < 0.01$) but not for the other responses. From bulls A and B, differences in post-thaw motility between sorted and unsorted sperm were near zero; for bull C, sorted sperm were 10 percentage points (19%) lower in motility than control sperm.

Table 8. Effect of sorting on post-thaw motility (%) and acrosomal status (%).

	Acrosomal status			Post-thaw motility
	Intact	Damaged	Non-intact	
Control	64 ^a	20 ^a	15 ^a	50 ^a
Sorted	65 ^a	14 ^b	21 ^b	46 ^b

^{a,b} Column means with different superscripts differ ($P < 0.05$).

6. Conclusion. Visual estimates of progressive motility for sorted, frozen sperm on average were slightly lower (4 percentage points; 8%) than for control sperm, although this difference was larger for one bull. These evaluations were made approximately 10 minutes after thawing. The small average difference is consistent with that for non-intact acrosomes after 2 h of incubation. Sperm with a damaged or missing acrosome are likely to be immotile. The increased percentage of sperm with a non-intact acrosome, for sorted samples, indicates damage associated with sorting or with cryopreservation before or after actual sorting. Presumably, sorting converted damaged acrosomes to missing acrosomes. Based on standard procedures for evaluation of sperm quality, there is no basis for assuming that fertilizing potential of these flow-sorted sperm should be severely compromised for most bulls.

Example 7

Sex-Selection and Cryopreservation of Bull Sperm Using 20% Egg Yolk-Tris Extender

Objective: to provide a protocol for the cryopreservation of flow-sorted bull sperm.

- 5 1. Collection and Ejaculate Assessment. Collect and prepare ejaculates as described in Example 1A. Select ejaculates from those bulls with >75% morphologically normal sperm. Visually estimate the percentage of progressively motile sperm (ejaculates that have progressive motility >60% are best for sorting). Add antibiotics to raw semen as follows: tylosin at a final concentration 100 µg/ml, gentamicin at a final concentration of 500 µg/ml, and linco-spectin at a
10 final concentration of 300/600 µg/ml.
2. Staining and Preparation for Sort. Following the addition of the antibiotics to the raw semen sample, allow 15-20 minutes before staining. Stain samples as described in Example 2.
15
3. Sorting. Sort for both X- and Y- type sperm, setting the sorting gates for 90% purity. Sort sperm into 50-ml Falcon tubes containing 2 ml 20% egg yolk-Tris A-fraction extender (see Example 2) until each tube contains a maximum of 20 ml total volume (or a maximum of 2 h per sort) and final sorted sperm concentration is 6×10^5 /ml. Note that
20 additional 20% egg yolk-Tris-A fraction catch buffer must be added after the sort and prior to cooling so that the final percentage of egg yolk is at least 3%.
4. Preparation for Freezing. Following the sort, cool the sorted samples to 5°C over a period of 90 minutes. After cooling, add 20% egg yolk-Tris B-fraction extender (see Example
25 2) stepwise (2X) at 15 minutes intervals. The final volume of Tris B-fraction extender added to the sperm sample should be equal to the volume of Tris A-fraction extender. The total volume of sperm sample after the Tris B-fraction extender is added should not exceed 27 ml total volume.

30 After the Tris B-fraction extender is added to the sperm sample, concentrate the sample by centrifugation for 20 minutes at $850 \times g$. Aspirate the supernatant leaving approximately 150 µl sperm pellet. Resuspend the sperm and pool the sperm for each individual bull.

5. Freezing. Add complete egg yolk-Tris extender (6% glycerol) to achieve a final sperm concentration of 20×10^6 /ml. Package the extended sperm into 0.25-ml polyvinylchloride straws for freezing as described in Example 2.

5

Example 8

Evaluation of the Fertility of Flow-sorted, Frozen Bull Sperm in Field Studies

MATERIALS AND METHODS

10 Semen Collection and Processing

Semen from young bulls of unknown fertility was collected via artificial vagina (see Example 1A). After determining sperm concentration with a spectrophotometer and subjective evaluation of progressive sperm motility, semen was processed and sorted as described in Example 2 except that the sperm were sorted by sex-type at 90% purity using a laser incident power of about 135 to about 150 mW. Processing and freezing was achieved as in Example 2 except that the equilibration time was about 3 h. Cornell Universal Extender (Seidel GE Jr., Theriogenology 1997; 48:1255-1264) was used for liquid semen in field trials 1, 2, and 3. For frozen semen in field trials 2 and 3, the extender used was 2.9% Na citrate + 20% egg yolk with a final glycerol concentration of 7% (see Example 1). For field trials 4 through 11, sperm were frozen in a Tris-based extender composed of 200 mM Tris, 65 mM citric acid, 56 mM fructose, 20% egg yolk, and a final glycerol concentration of 6% (see Example 2). The sheath fluid used in the flow cytometer was 2.9% Na citrate (see Example 4) for trials 1, 2, and 3, and a Tris buffer for the remaining trials (see Example 2).

Sperm were packaged in 0.25-ml French straws in columns as small as 50 μ l in the center of the straw. To minimize dilution effects, low volumes were used so there were at least 10^7 sperm/ml. In most trials, a column of extender without sperm was aspirated into the straw first to wet the cotton plug, followed by a small column of air, and then the sexed sperm. When sperm were frozen, one straw from each batch was thawed in 35°C water for 30 sec for quality control, and batches with less than 25% progressive motility post thaw were discarded. A sample of sexed sperm from each batch was sonicated and analyzed by flow cytometry to determine the accuracy of sexing.

Heifer Management and Artificial Insemination

The heifers used were in 6 widely scattered production units with different management practices. Seasonal and breed differences contributed further to the heterogeneity of the experiments (Table 9). Insofar as possible, treatments and controls were alternated systematically within bulls within inseminators as heifers entered the insemination facilities.

Estrus was synchronized in one of 4 ways (Table 9): (1) 500 mg of melengesterol acetate (MGA) fed daily in 2.3 kg of grain for 14 days followed by an i.m. injection of 25 mg prostaglandin F₂α (Lutalyse, Upjohn, Kalamazoo, MI, USA) 17, 18 or 19 days after the last day of feeding MGA (MGA/PG); (2) a single injection of 25 mg of prostaglandin F₂α (PG); (3) 20 or 25 mg of prostaglandin F₂α injected i.m. at 12-day intervals (PG/PG) or (4) 50 or 100 µg of GnRH injected i.m., followed by 25 mg of prostaglandin F₂α 7 days later (GnRH/PG).

Heifers were inspected visually for standing estrus mornings and evenings, but inseminated only in the evenings after 16:00, approximately ½ or 1 day after onset of estrus. Insemination was either into the uterine body conventionally, or half into each uterine horn using atraumatic embryo transfer sheaths (IMV, Minneapolis, MN, USA). In the latter case, semen was deposited past the greater curvature of the uterine horn as far anterior as could be accomplished without trauma, identically to nonsurgical embryo transfer. In most cases, semen was deposited between the anterior third and mid-cornua.

Most experiments included a frozen sperm control inseminated into the uterine body with 20 or 40 x 10⁶ sperm/dose from the same bulls used for sperm sorted for sex-type ("sexed"). This control served as a composite estimate of the intrinsic, normal fertility of the heifers under the specific field-trial conditions as well as the fertility of the bulls used and the skills of the inseminators. Some trials also included a low-dose, unsexed control group. Sometimes numbers of control inseminations were planned to be ½ or ⅔ the number used for each treatment to obtain more information on sexed sperm. Frozen sexed and control sperm were thawed for 20 to 30 sec in a 35 to 37°C water bath. Various other details are summarized in Table 9.

Pregnancy was diagnosed by ultrasound 28 to 37 d post insemination and/or 56 to 92 d post-insemination, at which time fetal sex was determined in most trials, as described in Curran, S., Theriogenology 1991; 36:809-814, without the operator's knowing insemination treatments or controls. Sexes of calves born were nearly identical to the fetal-sex diagnosis. Data were

analyzed by single-degree-of-freedom Chi square corrected for continuity; 2-tail tests were used unless 1-tail is specified. Fewer than 5% of the inseminations were culled due to errors of insemination treatment, frank infection of the reproductive tract, failure to traverse the cervix, etc. Decisions to cull animals from experiments were made shortly after insemination and were
5 never based on the pregnancy diagnosis.

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Table 9. Procedural details of field trials

	Trial	Insemination dates	Breeds of heifers	Bulls used	Inseminator s	Estrus synchroni- zation	Comments
5	1	5/20-23, 1997	Angus	N1, N2, AN4	A, B	MGA/PG	Included low-dose controls
	2	2/18-5/22, 1998	Angus crossbred	N3, N4, N5, N6	C, D	PG/PG	Low dose but no normal- dose controls; some heifers pregnant and aborted when synchronized
10	3	6/2-6/5, 1998	Angus	AN4, AN5, N7, N8	B, D	MGA/PG	
	4	2/10-13, 1999	Holstein	J2, J4	C, D	PG	Very severe mud, snow, wind, and cold, driving rain
15	5	2/24-26, 1999	Holstein	J2, J4, J5	C, B, D	PG/PG	
	6	4/14-16, 1999	Holstein	J2, J3, J4, J5	C, D	PG	Some heifers were reproductive culls
20	7	4/27-5/1, 1999	Hereford & Angus crossbred	AN1, AN4	C	MGA/PG	Semen for 1 bull shipped 6 h before sorting; severe weather
	8	4/21-23, 1999	Angus crossbred	H1, H2	E	MGA/PG	Feedlot heifers
25	9	5/5-8, 1999	Red Angus	AR1, AR2	C, F	MGA/PG	
	10	5/31-6/2, 1999	Angus	AN4, AN7, AN8	B, D	GnRH/ PG	
30	11	7/28-30, 1999	Holstein	H2, H3	C, D	PG/PG	First replicate available in a much larger trial

RESULTS AND DISCUSSION

The data presented are from 11 consecutive, heterogeneous field trials, constrained by logistical aspects of the studies, such as having to match bulls to genetic needs of the herds, unavailability of fertility information on bulls, limited numbers of heifers, unavailability of the same inseminators across trials, severe weather in some trials, limited amounts of sexed semen in early trials, 2 sets of heifers in which some turned out to be pregnant up to about 55 days at the time of estrus synchronization, etc. Up to 4 bulls and 3 inseminators were involved with each trial; this enabled us to sample populations to ensure that results applied to more than one bull or technician; however, insufficient data were produced to evaluate bull-to-bull differences in fertility rigorously.

Most sets of heifers were from breeding herds located 140 to 250 km from our laboratory. There were no significant differences in pregnancy rates between inseminators in any trial, but numbers of breedings per inseminator were low, and differences likely would be detected with larger numbers of inseminations.

Estrus synchronization methods were not compared within trials, so it was not possible to compare pregnancy rates among these methods. Pregnancy rates appeared to be satisfactory for all four synchronization procedures used.

Since inseminations were done once a day, heifers in estrus evenings were inseminated approximately 24 h after estrus was detected. The pregnancy rate for these heifers with sexed sperm pooled over all trials was 203/414 (49.0%), which was not significantly different ($P>0.1$) from that of heifers in estrus mornings and thus inseminated half a day after estrus detection 266/586 (45.4%). This tendency for higher fertility with later insemination is in agreement with findings from other research that it is preferable to inseminate later than normally recommended with lower fertility bulls, when low sperm numbers are used, or when conditions are otherwise suboptimal.

Pregnancy rates by treatments and, when available, fetal or calf sex are presented in Tables 10 to 20. The objective was to obtain female offspring, except in trial 8; accuracy was 95%, 83%, 90%, 83%, 82%, and 94% in Trials 1, 3, 8, 9, 10, and 11, respectively. In the remainder of the trials, fetal or birth sexes were not available because of timing of pregnancy diagnosis, unavailability of persons skilled in sexing fetuses, and/or because calves have not yet

been born. This was not a major concern because the main objective of this research was to determine fertility of flow-sorted sperm inseminated at low doses.

The accuracy of sexing can be adjusted to virtually any level desired between 50 and 95+% by adjusting the sorting parameters. However, higher accuracy results in lower numbers of sperm sorted per unit time, particularly for Y-chromosome sperm. 90% accuracy is sufficient for routine work.

The main findings from each field trial will be summarized in turn. Note that total sperm numbers are given in table headings; numbers of progressively motile sperm usually were 30 to 50% of these values. Field trial 1 (Table 10) confirmed that pregnancy rates with uterine horn insemination using low numbers of unsexed sperm were similar to controls with normal sperm numbers. The day 64 to 67 pregnancy rate with unfrozen sexed sperm (42%) was 12 percentage points below the unsexed liquid control with sperm diluted, stained, and centrifuged identically to the sorted sperm. Accuracy of sexing was 95%; the sex of calves born from sexed sperm matched the sex diagnosis of fetuses exactly; there was one mistake in sexing fetuses of controls. There were no abortions between 2 months of gestation and term, and all 19 calves from the sexed sperm treatment were normal and survived. For the sexed semen treatment, the 2-month pregnancy rates for bulls N1, N2, and N3 were 41, 44, and 40%, respectively; 39% (13/33) of heifers in estrus in the morning and 50% (6/12) in estrus in the evening became pregnant.

Table 10. Results of field trial 1 -- Angus heifers in Wyoming, 1997

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 33	No. pregnant day 64 to 67	No. ♀ calves
Sexed, 5°C/horns	3×10^5	45	20 (44%)	19 (42%)	18 (95%) ^a
Control, 5°C/horns	3×10^5	28	15 (54%)	15 (54%)	5 (53%) ^b
Frozen, control/body	40×10^6	29	16 (55%)	15 (52%)	11 (73%) ^{a,b}

^{a,b} Sex ratios without common superscripts differ ($P < 0.02$).

Field trial 2 (Table 11) provided the first evidence that results with sexed, frozen sperm are similar to sexed, unfrozen sperm if adjustment is made for numbers of sperm killed during cryopreservation. There also was no difference in pregnancy rates between sexed sperm stored at 5 versus 18°C. Pregnancy rates at 2+ months after insemination for sexed semen from individual bulls ranged from 22 to 42% pregnant ($P>0.05$). Embryonic loss between 1 and 2 months of gestation was very similar for sexed and control pregnancies. Calving data were available from 39 heifers from this trial; each of these heifers (30 sexed pregnancies, 9 controls) pregnant at 2 months calved after a normal-length gestation.

Table 11. Results of field trial 2--Crossbred beef heifers in Colorado, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 35 ^a	No. pregnant day 59 to 92 ^a
Control, 5°C/horns	5×10^5	58	27 (47%)	24 (41%)
Sexed, 5°C/horns	5×10^5	51	17 (33%)	16 (31%)
Sexed, 18°C/horns	5×10^5	46	16 (35%)	12 (26%)
Sexed, frozen/horns	1×10^6	87	29 (33%)	28 (32%)

^a No significant differences, χ^2 .

Field trial 3 (Table 12) confirmed that sexed, frozen sperm results in reasonable pregnancy rates. The accuracy of sexing sperm was confirmed again; however, there were 4 errors in sexing fetuses relative to the calves born; the actual sexes of calves born are presented. Again, there were no abortions between 2 months of gestation and term. Pregnancy rates averaged over sexed, unfrozen and sexed, frozen sperm for bulls N8, N9, AN5, and AN4 were 24, 31, 50, and 60%, respectively ($P<0.1$).

Table 12. Results of field trial 3 -- Angus heifers in Wyoming, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 62 to 65	No. ♀ calves
Sexed, 18°C/horns	5 x 10 ⁵	37	11 (30%) ^a	10 (91%) ^c
Sexed, frozen/horns	1 x 10 ⁶	35	18 (51%) ^{a,b}	14 (78%) ^c
Frozen, control/body	40 x 10 ⁶	37	27 (73%) ^b	16 (59%) ^d

^{a,b} Means without common superscripts differ P<0.05).

^{c,d} The percentage of ♀ calves from the sexed treatments (83%) differed from the control group, P<0.05, 1-tail, χ^2 .

Field trials 4, 5, and 6 (Tables 13, 14, 15) were done at the same location with 3 different groups of heifers. Unfortunately, it was not possible to replicate each trial similarly due to vagaries of field trials, such as scheduling personnel, availability of sexed semen from each bull, etc. The widely different pregnancy rates between trials 5 and 6 illustrate that conditions were different among trials. Some of the heifers in trial 6 were available because they failed to get pregnant after a month of natural mating. Under conditions of these trials, pregnancy rates were very similar between 1.5 and 3.0 x 10⁶ sexed, frozen sperm/dose. Furthermore, there was no advantage to uterine-horn insemination. There were no significant differences (P>0.05) in pregnancy rates among bulls except in Trial 5 in which the pregnancy rate of J2, 20/28 (71%), was higher than that of J4, 15/39 (38%) (P<0.05). This difference was not consistent from trial to trial, as J4 had numerically but not significantly (P>0.1) higher pregnancy rates than J2 in Trials 4 and 6.

Table 13. Results of field trial 4 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 33	No. pregnant day 64 to 67*
Sexed, frozen/body	1.5 x 10 ⁶	55	36 (65%) ^{a,b}	36 (65%) ^{a,b}
Sexed, frozen/body	3 x 10 ⁶	52	27 (52%) ^a	26 (50%) ^a
Control, frozen/body	20 x 10 ⁶	55	45 (82%) ^b	43 (78%) ^b

^{a,b} Means without common superscripts differ (P<0.01).

* Six heifers pregnant at d 30 to 33 were sold before the second pregnancy diagnosis; these were assumed to have remained pregnant.

Table 14. Results of field trial 5 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 35 ^a	No. pregnant day 60 to 62 ^a
Sexed, frozen/body	1.5 x 10 ⁶	23	12 (52%)	12 (52%)
Sexed, frozen/body	3.0 x 10 ⁶	25	15 (60%)	14 (56%)
Sexed, frozen/horns	1.5 x 10 ⁶	25	15 (60%)	12 (48%)
Sexed, frozen/horns	3.0 x 10 ⁶	25	17 (68%)	15 (60%)
Control, frozen/body	20 x 10 ⁶	30	20 (67%)	19 (63%)

^a No significant differences.

Table 15. Results of field trial 6 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 34	No. pregnant day 60 to 63
Sexed, frozen/body	1.5×10^6	27	11 (41%) ^a	9 (33%) ^a
Sexed, frozen/body	3.0×10^6	25	10 (40%) ^a	9 (36%) ^a
Sexed, frozen/horns	1.5×10^6	24	8 (33%) ^a	7 (29%) ^a
Sexed, frozen/horns	3.0×10^6	24	10 (42%) ^a	8 (33%) ^a
Control, frozen/body	20×10^6	24	18 (75%) ^b	17 (71%) ^b

^{a,b} Means without common superscripts differ ($P < 0.05$).

For trial 7 (Table 16), only one inseminator was available due to rescheduling. This is the only trial that showed a convincing advantage of uterine-horn over uterine-body insemination. For this inseminator under the conditions of the trial, 55% more heifers (22 percentage points) became pregnant with sexed, frozen semen inseminated into the uterine horns than into the uterine body. The true difference could be smaller because there are wide confidence intervals on these means. In all the other trials (5, 6, 9, and 11) in which body- and horn-insemination were compared, pregnancy rates were very similar for both methods for this technician as well as for other technicians.

Semen from one of the bulls used in Trial 7 was shipped without dilution from Montana by air in an insulated box at $\sim 20^\circ\text{C}$ before sorting; shipping time was 6 h. Pregnancy rates for the sexed sperm from the two bulls were virtually identical, 49% for the unshipped and 52% for the shipped semen. Semen was not diluted with extender and not cooled for shipping because staining properties of sperm with Hoechst 33342 are altered by dilution with extenders. Furthermore, in other studies (see Example 4), storing semen neat at ambient temperature between collection and flow-sorting was found to be superior to diluting it.

Table 16. Results of field trial 7 -- Crossbred beef heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 37
Sexed, frozen/body	1.5×10^6	86	34 (40%) ^a
Sexed, frozen/horns	1.5×10^6	86	53 (62%) ^b
Control, frozen/body	20×10^6	35	18 (51%) ^{a,b}

^{a,b} Means without common superscripts differ ($P < 0.01$).

Field trial 8 (Table 17) concerned feedlot heifers not implanted with growth promotants; at the time pregnancy was diagnosed they were aborted, so calving data was not available. This experiment illustrates that efficacious sexing also can be done in the male direction. Pregnancy rates for the 2 bulls were 50 and 61%.

Table 17. Results of field trial 8 -- Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant ^a day 74 to 76	No. ♂ fetuses
Sexed, frozen, 72 mW laser/body	1×10^6	18	7 (39%)	6 (86%)
Sexed, frozen, 135 mW laser/body	1×10^6	18	13 (78%)	12 (92%)

^a No significant differences.

Field trial 9 (Table 18) was the only trial to show a convincing advantage of 3.0 versus 1.5×10^6 sexed, frozen sperm/insemination dose. This advantage was true for both inseminators. Pregnancy rates for sexed sperm from the 2 bulls were 62 and 75%.

5 Table 18. Results of field trial 9 -- Red Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 60 to 63 ^a	No. ♀ fetuses
Sexed, frozen/body	1.5×10^6	15	8 (53%)	7 (88%)
Sexed, frozen/body	3.0×10^6	14	12 (86%)	9 (75%)
Sexed, frozen/horns	1.5×10^6	16	9 (56%)	7 (78%)
Sexed, frozen/horns	3.0×10^6	16	12 (75%)	11 (92%)
Control, frozen/body	20×10^6	30	21 (70%)	13 (62%)

10 ^a 3.0×10^6 sexed sperm had a higher pregnancy rate (80%) than 1.5×10^6 sexed sperm (55%), $P < 0.05$, 1-tail χ^2 .

15 Pregnancy rates in field trial 10 (Table 19) with sexed, frozen semen, were similar to controls; the accuracy of sexing sperm on this trial was only 82%, which, however, is not significantly different from the targeted 90% accuracy. Pregnancy rates for sexed semen were 54, 66, and 50% for bulls AN4, AN7, and AN8, respectively ($P > 0.1$). Eighteen of the heifers
 20 inseminated in this trial were the calves resulting from sexed sperm in field trial 1.

Table 19. Results of field trial 10 -- Angus heifers in Wyoming, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 61 to 63 ^a	No. ♀ fetuses
Sexed, frozen/body	1×10^6	44	26 (59%)	23 (85%)
Sexed, frozen/body	3×10^6	43	23 (53%)	17 (74%)
Control, frozen/body	20×10^6	35	20 (57%)	12 (57%)

25 ^a No significant differences.

30

Table 20. Results of field trial 11 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 28 to 30 ^a	No. pregnant day 56 to 58 ^{a,b}
5 Sexed, frozen/body	1 x 10 ⁶	12	8 (67%)	7 (58%)
Sexed, frozen/body	3 x 10 ⁶	12	6 (50%)	4 (33%)
Sexed, frozen/horns	1 x 10 ⁶	7	4 (57%)	4 (57%)
Sexed, frozen/horns	3 x 10 ⁶	7	4 (57%)	4 (57%)
10 Control, frozen/body	20 x 10 ⁶	9	4 (44%)	3 (33%)

^a No significant differences, χ^2 .

^b 16 of 17 (94%) fetuses from the sexed semen treatments were female; 2 were too deep in the body cavity to sex with ultrasound.

Data from trials were combined in which treatments were identical except 1 x 10 and 1.5 x 10⁶ sperm doses were pooled (Table 21).

Table 21. Meta-summary from combining trials with sexed, frozen semen and frozen controls.

Trials combined	Sperm no./site	No. heifers	No. pregnant
5, 6, 9, 11	1.0 - 1.5 x 10 ⁶ /body	77	36 (47%)
	3.0 x 10 ⁶ /body	76	38 (50%)
	1.0 - 1.5 x 10 ⁶ /horns	72	32 (44%)
	3.0 x 10 ⁶ /horns	72	39 (54%)
	20 x 10 ⁶ /body, control	93	61 (66%)
4, 5, 6, 9, 10, 11	1.0 - 1.5 x 10 ⁶ /body	176	98 (56%)
	3.0 x 10 ⁶ /body	171	88 (51%)
	20 x 10 ⁶ /body, control	183	124 (68%)
25 5, 6, 7, 9, 11	1.5 x 10 ⁶ /body	163	70 (43%)
	1.5 x 10 ⁶ /horn	158	85 (54%)
	20 x 10 ⁶ /body, control	128	79 (62%)

Pregnancy rates with sexed sperm were generally 70-90% of unsexed controls within experiments with 7 to 20 times more sperm. This difference was less in the more recent trials, possibly reflecting improved sexing and sperm-processing procedures.

In some trials, heifers were examined for pregnancy by ultrasound at both 1 and 2 months after insemination. Pregnancy losses in this interval were similar ($P>0.1$) for sexed (23/261; 8.8%) versus control (9/145; 6.2%) sperm treatments, which is one measure that genetic damage due to sexing is minimal. Calving information was obtained from only a few of the pregnant heifers because most cattle from the earlier trials were sold, and those from later trials have not calved yet. The population of calves produced to date from sexed semen appears to be no different from the population of controls.

CONCLUSION

Sex ratios in cattle can be distorted to about 90% of either sex by sorting sperm on the basis of DNA content with a flow cytometer/cell sorter followed by cryopreservation and relatively routine artificial insemination. Calves resulting from sexed sperm appear to be normal. For most bulls in these studies, pregnancy rates with 1.0 to 1.5×10^6 sexed, frozen sperm were 70 to 90% of unsexed controls with 20 or 40×10^6 frozen sperm inseminated conventionally. These results apply to well-managed heifers bred by well-trained inseminators using properly processed semen. There may be a small advantage to inseminating sexed sperm bilaterally into the uterine horns compared to standard uterine body insemination.

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the ends of the present invention.

All patents and publications described are herein incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

- 1 1. A method for the cryopreservation of sperm comprising:
- 2 (a) obtaining a selected sperm sample;
- 3 (b) cooling said selected sperm sample;
- 4 (c) isolating sperm from said selected sperm sample to produce isolated sperm;
- 5 (d) adding final extender to said isolated sperm to produce a suspension of
- 6 sperm; and
- 7 (e) freezing said suspension of sperm.
- 1 2. The method of Claim 1 wherein said selected sperm sample comprises a portion of the
- 2 sperm present in a source sample, said portion of sperm selected for a characteristic, and
- 3 wherein the sperm concentration in the selected sperm sample is lower than in the source
- 4 sample.
- 1 3. The method of Claim 1 wherein said selected sperm sample comprises sex-selected
- 2 sperm.
- 1 4. The method of Claim 1 wherein said selected sperm sample comprises mammalian
- 2 sperm.
- 1 5. The method of Claim 4 wherein said selected sperm sample comprises bovine sperm.
- 1 6. The method of Claim 4 wherein said selected sperm sample comprises equine sperm.
- 1 7. The method of Claim 4 wherein said selected sperm sample comprises porcine sperm.
- 1 8. The method of Claim 1 wherein said selected sperm sample comprises sperm selected by
- 2 a method from the group consisting of flow cytometry, a magnetic technique, a columnar
- 3 technique, a gravimetric technique, a biochemical technique, a technique based on

4 motility of sperm, a technique based on an electrical property of sperm, and any
5 combination thereof.

1 9. The method of Claim 8 wherein said sperm have been selected by flow cytometry.

1 10. The method of Claim 1 wherein cooling is carried out by reducing the temperature of the
2 selected sperm sample to about 5°Celsius.

1 11. The method of Claim 10 wherein cooling is carried out over a period of about 60 minutes
2 to about 240 minutes.

1 12. The method of Claim 1 wherein the final extender added to said selected sperm sample
2 (each) comprise, in addition to a cryoprotectant, one or more of the following components:
3 a component that maintains osmolality and buffers pH, an organic substance that reduces
4 cold shock and preserves fertility of sperm, an energy source, a substance that facilitates
5 sperm capacitation, and an antibiotic.

1 13. The method of Claim 12 wherein said cryoprotectant is selected from the group
2 consisting of disaccharides, trisaccharides, and any combination thereof.

1 14. The method of Claim 12 wherein said cryoprotectant is selected from the group
2 consisting of glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any
3 combination thereof.

1 15. The method of Claim 12 wherein said component that maintains osmolality and buffers
2 pH is selected from the group consisting of a buffer comprising a salt, a buffer containing
3 a carbohydrate, and any combination thereof.

1 16. The method of Claim 12 wherein said component that maintains osmolality and buffers
2 pH is selected from the group consisting of sodium citrate,
3 Tris[hydroxymethyl]aminomethane,

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- 4 N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, monosodium glutamate,
5 milk, HEPES buffered medium, and any combination thereof.
- 1 17. The method of Claim 12 wherein said organic substance is selected from the group
2 consisting of egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin,
3 and any combination thereof.
- 1 18. The method of Claim 12 wherein said energy source is a monosaccharide selected from
2 the group consisting of glucose, fructose, mannose, and any combination thereof.
- 1 19. The method of Claim 12 wherein said antibiotic is selected from the group consisting of
2 tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin,
3 and any combination thereof.
- 1 20. The method of Claim 1 wherein, after the addition of the final extender, the sperm sample
2 and suspension of sperm, respectively, comprise glycerol, sodium citrate,
3 Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.
- 1 21. The method of Claim 1 wherein, after the addition of the final extender, said sperm
2 sample and suspension of sperm, each comprise glycerol, sodium citrate, egg yolk, and
3 one or more antibiotics.
- 1 22. The method of Claim 1 wherein, after the addition of the final extender, said sperm
2 sample and suspension of sperm, each comprise glycerol, egg yolk, milk, fructose, and
3 one or more antibiotics.
- 1 23. The method of Claim 1 wherein said extender has a pH in the range of about 6.5 to about
2 7.5.
- 1 24. The method of Claim 1 wherein the sperm are isolated from said selected sperm sample
2 by centrifugation.

any
one
from this
claim.

See
Claim
12.
phage
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room

1 25. The method of Claim 24 wherein said centrifugation allows for at least about 50% to
2 about 90% recovery of sperm.

1 26. The method of Claim 1 wherein the concentration of sperm in said suspension prior to
2 freezing is about $1 \times 10^6/\text{ml}$ to about $300 \times 10^6/\text{ml}$.

1 27. A frozen selected sperm sample comprising a portion of the sperm present in a source
2 sample, said portion of sperm selected for a characteristic.

II 1 28. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 comprises sex-selected sperm.

1 29. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 comprises mammalian sperm.

1 30. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises bovine sperm.

1 31. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises equine sperm.

1 32. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises porcine sperm.

1 33. The frozen selected sperm sample of Claim 27 wherein the method used to select said
2 selected sperm sample comprises a technique from the group consisting of flow
3 cytometry, a magnetic technique, a columnar technique, a gravimetric technique, a
4 biochemical technique, a technique based on motility of sperm, a technique based on an
5 electrical property of sperm, and any combination thereof.

1 34. The frozen selected sperm sample of Claim 33 wherein said frozen selected sperm sample
2 comprises sperm that have been selected by flow cytometry.

1 35. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 is produced by a method comprising:

- 3 (a) obtaining a selected sperm sample;
4 (b) cooling said selected sperm sample;
5 (c) isolating sperm from said selected sperm sample to produce isolated sperm;
6 (d) adding final extender to said isolated sperm to produce a suspension of
7 sperm; and
8 (e) freezing said suspension of sperm.

1 36. A method comprising using the frozen selected sperm sample of Claim 27 for artificial
2 insemination or in vitro fertilization.

1 37. The method of Claim 36 comprising using said frozen selected sperm sample for low-
2 dose artificial insemination.

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ABSTRACT OF THE INVENTION

The present invention provides a method of cryopreserving sperm that have been selected for a specific characteristic. In a preferred embodiment, the method is employed to freeze sex-selected sperm. Although the cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred. The present invention also provides a

The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or bison sperm. The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization.

the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

fertilization.

$\frac{d}{dt} \left(\frac{\partial L}{\partial \dot{x}} \right) = \frac{\partial L}{\partial x}$

Listing of Claims:

Claims 1-37 (Canceled)

38. (Previously Presented)

A method of cryopreserving sex-selected sperm cells, comprising:

- a. obtaining sperm cells from a species of a male mammal;
- b. sorting said sperm cells, without the presence of protective compounds in seminal plasma, and based upon sex-type to create a collection of sex-selected sperm cells;
- c. cooling said sex-selected sperm cells;
- d. suspending said sex-selected sperm cells in an extender to at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender, and;
- e. freezing said sex-selected sperm cells in said extender.

39. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal are selected from the group consisting of bovine sperm cells and equine sperm cells.

40. (Withdrawn)

A method of cryopreserving sperm cells as described in claim 39, and further comprising the step of isolating a number of bovine sperm cells between about 300,000 and about 3,000,000.

41. (Withdrawn)

A method of cryopreserving sperm cells as described in claim 39, and further comprising the step of isolating a number of bovine sperm cells of no more than about 1,000,000.

42. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal comprise equine sperm cells.
43. (Currently amended)
A method of cryopreserving sex-selected sperm cells as described in claim 42, and further comprising the step of isolating a number of equine sperm cells between about 1,000,000 ~~million~~ and about 25,000,000.
44. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 42, and further comprising the step of isolating a number of equine sperm cells of no more than about 5,000,000.
45. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said step of cooling sex-selected sperm cells comprises reducing the temperature of said sex- selected sperm cells to about 5°Celsius.
46. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 45, wherein said step of reducing the temperature of said sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells for a period of about 60 minutes to about 240 minutes.
47. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said extender further comprises a component which maintains osmolality and buffers pH.
48. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 47, wherein

said component which maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.

49. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris[hydroxymethyl]aminomethane, 200mM Tris[hydroxymethyl]aminomethane, 175 mM to 225mM Tris[hydroxymethyl]aminomethane, 200 mM Tris[hydroxymethyl]aminomethane/65mM citric acid monohydrate, 175 mM to 225mM Tris[hydroxymethyl]aminomethane/50mM to 70mM citric acid monohydrate, N-Tris [hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/65 mM citric acid monohydrate, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/50mM to 70 mM citric acid monohydrate, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

50. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, 48, or 49, wherein said extender has a pH in the range of about 6.5 to about 7.5.

51. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 50, wherein said extender further comprises a cold shock protectant.

52. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 51, wherein said cold shock protectant is selected from the group consisting of egg yolk, 20% egg

yolk, 15% to 25% egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.

53. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 51, wherein said extender further comprises an energy source.

54. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 53, wherein said energy source is selected from the group consisting of a saccharide, glucose, fructose, 56 mM fructose, 45mM to 60mM fructose, mannose, and any combination thereof.

55. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 53, wherein said extender further comprises an antibiotic.

56. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 55, wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.

57. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, 51, 53, or 55, wherein said extender further comprises a cryoprotectant.

58. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.

59. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of glycerol, 6% glycerol, between 5% to 7% glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.

60. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

61. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, egg yolk, and one or more antibiotics.

62. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, egg yolk, milk, fructose, and one or more antibiotics.

63. (Currently amended)

A method of cryopreserving sex-selected sperm cells as described in claim 38, further comprising ~~the step~~ a step of equilibrating said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to said freezing step for a period of about 1 hour to about 18 hours.

64. (Currently amended)

A method of cryopreserving sex-selected sperm cells as described in claim 59, further comprising ~~the step~~ a step of equilibrating said sex-selected sperm cells suspended in said

extender to a cooler, non-freezing temperature for a period of time prior to said freezing step over a period of not greater than 6 hours.

65. (Withdrawn)

A frozen sex-selected sperm sample in accordance with the method of claim 38.

66. (Canceled)

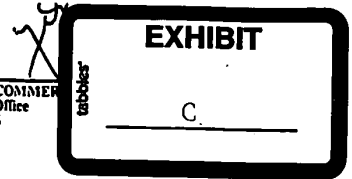
67. (Withdrawn)

A method of cryopreserving sperm cells as described in claim 38, wherein said step of freezing said sex-selected sperm cells in said extender comprises freezing a number of bovine sperm cells between about 300,000 and about 5,000,000.



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09/478,299	01/05/2000	John L. Schenk	22091-701	1509

7590 05/13/2005

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EXAMINER

MELLER, MICHAEL V

ART UNIT	PAPER NUMBER

1654

DATE MAILED: 05/13/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No. 09/478,299		Applicant(s) SCHENK, JOHN L	
	Examiner Michael V. Meller		Art Unit 1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on 28 February 2005.

2a) ☒ This action is FINAL. 2b) ☐ This action is non-final.

3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4) ☒ Claim(s) 38-65 and 67 is/are pending in the application.

4a) Of the above claim(s) 40, 41, 55, 56, 60-62, 65 and 67 is/are withdrawn from consideration.

5) ☐ Claim(s) _____ is/are allowed.

6) ☒ Claim(s) 38, 39, 42-54, 57-59, 63, 64 is/are rejected.

7) ☐ Claim(s) _____ is/are objected to.

8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9) ☐ The specification is objected to by the Examiner.

10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
 a) ☐ All b) ☐ Some * c) ☐ None of:
 1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892) 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948) 3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.	4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____. 5) ☐ Notice of Informal Patent Application (PTO-152) 6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

The election/restriction of record is maintained.

Claims 40, 41, 55, 56, 60-62, 65, 67 are withdrawn from further consideration as being drawn to non-elected subject matter.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claim Rejections - 35 USC § 112

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

There is still no support in the specification for the ranges of sperm cells claimed. Applicant has not addressed this rejection but it is still valid. There is no support for limitations such as, "to at least about 5 million per milliliter to at least about 10 million

per milliliter of extender", "equine sperm cells between about 1,000,000 million and about 25,000,000".

The specification may mention specific points of the concentration (i.e. 1×10^6) but it does not provide for the claimed range of the concentrations (i.e. between about 1,000,000 million and about 25,000,000). The concentrations in table 1 are noted but they are only isolated concentrations, there is no support for ranges of these concentrations, only the specific points tested.

The time limitation still has no support. In fact, as applicant noted, they have support for 1-18 hours, which clearly is more than 6 hours.

Applicant points to the specification at page 17, line 6, but this is not what the problem is. There is no support for "over a period of not more than 6 hours". This is because 1-18 hours clearly is more than 6 hours.

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 63 and 64 are still confusing. The way the applicant has now amended the claims is confusing since it is not clear how the "at least some of said sex selected sperm cells" can be not frozen as in claims 63 and 64 and indeed frozen in the main claim, claim 38. If claim 38 requires that the "at least some of said sex selected sperms cells" are to be frozen then claims 63 and claim 64 fail to further limit claim 38.

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It might be clearer if applicant states in claims 63 and 64 that the steps of claims 63 and 64 are performed between steps d) and e) of claim 38.

Further, what is meant by , "to at least about 5 million per milliliter to at least about 10 million per milliliter of extender" ? Is this the concentration of the extender before it is applied to the sperm cells ? Is the 5 million referring to the number of sperm cells. So how much extender is added to each sperm cell ?

This was never addressed by applicant.

Cooling is still not clear in claim 38. Cooling to what temperature ?

Claims 63 and 64 are confusing. First there is no antecedent basis for "the step of equilibrating at least some of said sex-selected sperm cells". What step is this ? Second, what temperature is cooler ? Cooler than what ? This is relative and subjective. There is no point of reference for one to compare it to.

Claim Rejections - 35 USC § 103

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. in view of Spaulding and Shrimpton.

The claims still are obvious over the cited prior art of record. Applicant's limitation in claim 38 of "sorting said sperm cells, without the presence of protective compounds in seminal plasma" is met since as applicant notes on page 10 of their latest response filed 2/28/2005, that flow cytometry which is used by Spaulding has been shown to work and is now in practice as applicant has also done. Applicant states on page 10 that sorting

without the presence of protective compounds is a feature that occurs when flow cytometry is used to achieve sex sorting which Spaulding has also done just like applicant and this is of record. Thus, the references meet the claim limitations.

Applicant also argues that the concentrations of the sperm cells in the extenders is 5 million to 10 million and applicant has pointed to table 1 of the specification to support this, but these points go up and down. For example, the values go up as one approaches 10 million and then decreases as one goes above 10 million. Also, it is not clear what this signifies. It is not clear what significance the increase in the numbers means. Does this increase anything? Just because one yields higher numbers at 10 million this does not correlate to anything and if it does what does it correlate to? Further, it is not clear what the 61 refers to in relation to 10 million in table 1. Also it is noted that the values at 24 hours and 48 hours differ in some way. It is not clear what this signifies.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire **THREE MONTHS** from the mailing date of this action. In the event a first reply is filed within **TWO MONTHS** of the mailing date of this final action and the advisory action is not mailed until after the end of the **THREE-MONTH** shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any

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extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 571-272-0967. The examiner can normally be reached on Monday thru Thursday: 9:30am-6:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Bruce Campell can be reached on 571-272-0974. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).



Michael V. Meller

Application/Control Number: 09/478,299
Art Unit: 1654

Page 7

Primary Examiner
Art Unit 1654

MVM

EXHIBIT

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SECOND EDITION

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Physiology of Reproduction and Artificial Insemination of Cattle

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SAN FRANCISCO



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16

Extenders and Extension of Unfrozen Semen

16-1 INTRODUCTION

The concentration of spermatozoa in normal, freshly ejaculated bull semen is high. Considerable extension* is necessary to provide a convenient volume to inseminate that will contain enough sperm cells to ensure maximum fertilization rates without wasting spermatozoa. Mixing spermatozoa with extender allows the addition of many ingredients that sustain and protect the spermatozoa, thereby preserving fertility until they are used for insemination. Thus the two major functions of semen extenders are to preserve the fertility of sperm cells and to increase the total volume so that the proper dose of cells for insemination can be conveniently packaged and used. A single ejaculate can therefore be used to inseminate several thousand cows.

Spermatozoa must be alive to be fertile and motility has been used to monitor viability. Motility, however, is primarily a reflection of flagellar activity, and it does not guarantee that such cells are fertile. The integrity of the genetic material in the sperm chromatin must also be preserved in order for normal embryo development to take place after fertilization. Extending media and storage conditions should preserve both functions but there is no test of semen quality prior to use that can predict how well the genetic material is maintained.

There are countless recipes for media that will preserve bull spermatozoa, several of which are described in subsequent sections. Much of the early work, particularly in Russia, has been reviewed by Anderson (1). Physiological salt solutions were found to be unsuitable for preserving sperm cells. Glucose, sulfate, and tartrate were often included in the Russian formulations, and Milovanov and Selivanova (2) reported that lecithin and peptone protected the lipid membranes of spermatozoa. None of the media, however, appreciably extended the fertile storage life of bull spermatozoa.

*"Extension" and "extender" are considered preferable to "dilution" and "diluter," since the material added to semen actively extends the life of the sperm cells.

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A major breakthrough in semen extenders came when Phillips (3) reported the value of hen's egg yolk. In the original formula the extender contained equal volumes of egg yolk and phosphate buffer. Salisbury et al. (4) showed that, by replacing the phosphate with sodium citrate, equal fertility, higher spermatozoan motility during prolonged storage, and improved visibility resulted. The latter of course made microscopic evaluation easier. The yolk-citrate extender, with various modifications, has been the most widely used medium for artificial insemination in cattle. A variety of extenders have been formulated for unfrozen spermatozoa (5).

Proper identification of the bull that produced each sample of semen is extremely important. Hence all containers used from semen collection to insemination must be properly labeled. Harmless vegetable dyes are added to the extender (6, 7) to facilitate the identification of breeds, or bulls where the number is limited. Different-colored straws are used for the same purpose with frozen semen. Color seems to be a convenient aid, but no data are available on its effectiveness in reducing identification errors.

16-2 PRINCIPLES OF SPERM PRESERVATION

16-2.1 Temperature. The storage time required for practical use of most semen in artificial breeding varies from a few hours to several weeks. Metabolic rate tends to be proportional to absolute temperature, so reduced temperature has been the chief means of slowing down chemical reaction and prolonging life. Spermatozoa can tolerate temperatures somewhat higher than body temperature for short periods of time, but energy requirements are high and toxic products can accumulate rapidly. Chemical inhibition of spermatozoa is helpful at ambient temperatures (see Section 16-4).

Most of the inseminations before the advent of frozen semen were performed with semen stored at about 5°C, but upon rewarming to 37°C the percentage of progressively motile cells was observed to have decreased during storage. Metabolism continues at a reduced rate at 5°C and fertility begins to decrease before day 4 (8, 9). When semen is frozen (see Chapter 17) spermatozoa may remain fertile for years, although certain characteristics of aging are evident in semen stored at -196°C (8, 10).

At the same time that temperature is decreased, changes occur in the internal and external environments of spermatozoa. The solubility of gases in the external environment increases. For example, when there is a decline in temperature from body temperature to 0°C, solubility of O₂ doubles and CO₂ triples. With air as the gas overlying the spermatozoa, this shift in solubility of gases at lower temperatures permits a relatively greater proportion of the total metabolic activity to be due to oxidative metabolism. At higher-than-body temperatures the solubility of oxygen is relatively low in relation to the rapid metabolic rate and may become self limiting under some circumstances, even at body temperature. At low temperatures the solubility of oxygen increases,

and the metabolic activity decreases several-fold, resulting in a relatively greater oxygen supply. The solubility of certain solutes decreases, and the physical form of the colloids in the semen can change as well, causing increased semen viscosity and therefore a greater physical resistance to motility.

As temperature decreases, spatial arrangements of the internal structure and outer covering of the cells may change, a contraction of protoplasm may occur and can happen at different rates depending on the chemical components of the various structures. All such factors, as well as the rates of chemical reactions, are involved in controlling the fertile life of sperm cells by temperature reduction.

16-2.2 Energy Source. The most obvious energy requirement of the sperm cell is for motility, but additional energy is presumably required for cell maintenance. Spermatozoa are capable of both aerobic and anaerobic metabolism. A simple source of energy such as glucose or fructose should be supplied in the extender to protect intracellular reserves and cell components. Fructose, present in the seminal plasma, will be diluted considerably by semen extension. Egg yolk contains some glucose and other compounds utilizable by bull spermatozoa.

16-2.3 Osmotic Pressure and Electrolyte Balance. Spermatozoa behave as osmometers and are capable of extensive changes in size depending upon the tonicity of the medium in which they exist (11, 12). Seminal plasma has an osmotic pressure of about 285 milliosmols and it is usually assumed that this represents the ideal physiological osmotic pressure. Even if this is true, however, it should not be assumed that solutions of equivalent osmotic pressure, as measured by physical tests, are isotonic with bull spermatozoa. The permeability of the cell to different substances varies. Interactions may occur between the total concentration of substances in the fluid surrounding spermatozoa, the pH of the medium, and the specific ions and nonelectrolytes present (13, 14). If the tonicity of the medium deviates considerably spermatozoa may have bent tails, swim in circles, and die. Fortunately spermatozoa are able to tolerate a moderate range of osmolarities without a reduction in fertility (15); the degree of tolerance is affected by interactions with pH and other ions present (13). Extenders developed to preserve sperm should ideally be isotonic under the conditions used, and thus should not cause morphological or functional damage.

Solutions that are similar in composition to body fluids or are naturally produced liquids, such as egg yolk and milk, appear to be compatible with sperm. Buffered egg yolk first developed for use with bull sperm in artificial insemination was hypertonic (16, 17). Subsequently the concentrations of the salt solutions used were reduced to isosmotic levels. More recently a series of zwitter ion buffers has been used (15, 18-22) and bull spermatozoa suspended in them appear to tolerate a range of osmolarities.

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16-24 Buffering and pH. Spermatozoa need protection from autotoxication due to acid products of metabolism, particularly when they are stored without refrigeration. Bull sperm motility and fertility are well preserved in egg yolk and milk extenders near neutral pH, although reduction of the pH to 6.5 may even be beneficial (23). The optimum pH probably varies with storage temperature and other components of the extender (13, 14). Saturation of bicarbonate media with CO_2 causes metabolic inhibition and reduces the pH to about 6.3 (24, 25, 26). This inhibition may be due in part to a reduction in pH (23).

16-25 Proper Gas Phase. To maintain the desired gas conditions in the extending medium, the relative proportion of gas to liquid phases in sealed containers and the composition of the gas phase should be controlled (25, 27-30). As previously stated, CO_2 inhibits spermatozoan metabolism, the degree of inhibition of anaerobic glycolysis being partially dependent upon CO_2 levels and sulfite present. Oxygen with suitable substrate can provide the sperm cells with an efficient energy source. However, toxic products such as H_2O_2 may be formed unless catalase is included (31). The effects of light are especially damaging in the presence of oxygen (31, 32, 33), and gassing with nitrogen is beneficial.

16-26 Inhibition of Microorganism Growth. Egg yolk and other extending media for bull spermatozoa provide a good environment for the growth of microorganisms, which produce products that may be harmful to spermatozoa, and which may infect the cow. Some organisms usually will be found in semen from healthy bulls even under aseptic semen collection and processing conditions. It is therefore standard practice to include antibiotics, such as penicillin and streptomycin, in all extenders used commercially (34, 35, 36). The various agents used, and their application, are discussed in Section 16-5.2.

16-27 Exclusion of Toxic Materials. Not only should extenders be formulated that will prevent the formation of toxic products during storage, but the extending media, as prepared, should be free of substances that are harmful to spermatozoa. Heavy metals, for example, are harmful to spermatozoa, but proper distillation of water insures that only nontoxic trace amounts remain. Only pure reagents should be used in formulating buffer (37). Unfortunately, cryoprotective agents such as glycerol (38, 39), required for maximum protection of spermatozoa during freezing, are somewhat toxic; and dead spermatozoa are a source of amino acid oxidase, which is harmful to surviving cells through the production of H_2O_2 (40).

16-28 Semen Extension and the "Dilution" Effect. Bull semen should be mixed with enough extender so that the initial sperm concentration will be

diluted to yield an optimum number of spermatozoa for insemination in a convenient volume. Studies have shown that extensive "dilution" of the spermatozoa with simple solutions depresses motility (41) but that the addition of amino acids and macromolecules such as egg albumin or casein minimizes this effect. Rottensten et al. (42) found that heated plasma mixed in equal volumes with a citrate-glucose solution improved the survival of sperm diluted up to 1:200 and stored at 4°C. Adding 25 percent egg yolk was as beneficial as the seminal plasma. Chemical inhibition can prolong motility of bull spermatozoa highly diluted with synthetic media (43). Bovine cervical mucus also counteracts the dilution effect (44).

In the experiments of Rottensten et al. (42), egg yolk and milk in practical extenders appear almost to prevent the dilution effect. Catalase, CO_2 , and N_2 (29, 45, 46) may all help to eliminate the dilution effect.

The procedure used to yield suitable doses of spermatozoa for insemination is described in Section 16-7.

16-2.9 Protection of Spermatozoa Against the Cold. When mammalian sperm cells are cooled to 5°C they are subject to cold shock, which causes leakage of intracellular enzymes, potassium, lipoprotein, ATP, and other materials from the cells. Furthermore, motility is decreased and the flagellum may become bent (41). The precise mechanisms of damage are unknown, but presumably changes occur at unequal rates on the surface and internal portions of spermatozoa during cooling, and both physical and chemical damage result. Ethylenediamine tetracetic acid (EDTA) may reduce cold shock (47); and if cells are cooled slowly, particularly below 20°C (48), damage is less severe, which indicates that spermatozoa can make adaptive changes.

However, the most effective means of protecting spermatozoa against the effects of chilling is to provide them with lecithin, proteins, lipoproteins, and similar complexes found in egg yolk or milk (49-54). The importance of adding egg yolk before cooling is clearly demonstrated by experiments that show a marked beneficial effect on subsequent motility and fertility (55).

The optimum cooling rate, with regard to fertility, for semen in different extenders has not been adequately established. Extremely rapid cooling should be avoided, but otherwise considerable latitude between 1 and 4 hours is possible. There seems to be little interaction between osmotic pressure of the extenders and optimum cooling rate (56).

16-3 EXTENDERS FOR REFRIGERATED SEMEN

16-3.1 Historical. The first media used to dilute semen were primarily salt or sugar solutions designed to increase the volume of semen for immediate use rather than to conserve it (1, 41). When the value of egg yolk was discovered (3) a "preserving" extender was developed that could be coupled with the advantages of reducing metabolic activity of spermatozoa and prolonging their fertility by storage at approximately 5°C.

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Semen extenders should be formulated that embody the principles of sperm preservation discussed previously. Furthermore, they should be easy to prepare, stable, and economical. Many extenders for liquid semen have been described and their development reviewed (1, 5, 20, 57, 58).

16-3.2a Egg Yolk Phosphate. In 1939 Phillips (3) first reported that fresh egg yolk combined with a phosphate buffer preserved the motility and fertility of refrigerated bull spermatozoa. The formula was: KH_2PO_4 , 0.2 gram; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 2.0 grams; glass-distilled water, 100 milliliters; hen's egg yolk, 100 milliliters. The final pH of this medium was 6.75. In field tests this extender gave good fertility (59).

16-3.2b Egg Yolk Citrate. In 1941 Salisbury et al. (4) reported that sodium citrate was beneficial to sperm liveability when it was added to an equal volume of egg yolk. Sodium citrate had the advantage over phosphate buffer in being a chelating agent and in dispersing the fat globules in egg yolk, thereby improving visibility when spermatozoa were examined microscopically. The fertility of bull semen in phosphate and citrate extenders appeared to be equal, as indicated by the following results:

Extension rate	Variable	Yolk-phosphate	Yolk-citrate	Reference
1:4	Services/conceptions	1.53	1.53	(4)
1:200	60-90-day % nonreturns (NR)	50.5	50.5	(60)

In these studies either 3.6 percent or 3.92 percent (w/v) of sodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) was used. This buffer concentration is hypertonic for bull spermatozoa but apparently, when combined with 50 percent egg yolk, did not cause osmotic problems. Later (16) the recommended concentration of sodium citrate dihydrate was reduced to 2.9 percent (w/v).

The citrate-yolk extender especially has been modified many times with varying concentrations of sodium citrate and egg yolk and the addition of glucose, sulfanilamide, and antibiotics (61). When sulfanilamide was included in the yolk-citrate extender a highly significant increase in fertility level—about five percentage units—was obtained (60, 62). This probably explains why van Dielen (63) obtained 54.6 percent NR (nonreturn) with a yolk-citrate extender containing sulfanilamide and only 50.8 percent NR ($P < 0.01$) with the phosphate extender in an experiment involving 7,101 inseminations. Also, egg yolk was reduced to 33 percent and thus the hyposmotic condition of the phosphate extender may have been deleterious (64). A nonreturn rate of 76.3 percent following insemination of 14,605 cows with sperm in a yolk-citrate-glucose extender was obtained, compared to 74.7 percent for 14,056 cows inseminated

with a yolk-phosphate extender (65). Foote and Bratton (66) also found yolk-citrate-sulfanilamide extender to be superior to yolk-phosphate. One trial (67) resulted in higher fertility for a yolk-phosphate extender than for yolk-citrate. When sulfanilamide and antibiotics were added the reverse was true, possibly because of an unfavorable antagonism between phosphate and streptomycin.

In general, yolk-citrate has been the standard against which new extenders have been compared. Salisbury and VanDemark (213) reported that glucose added to yolk-citrate increased the liveability of spermatozoa. Ohms and Willett (68) modified the 2.9 percent citrate-yolk extender by replacing part of the citrate with glucose. After one day of storage at 5°C the yolk-citrate-glucose had a 1.8 percent advantage in nonreturn rate ($P > 0.05$) and after two days of storage it had a 6.0 percent advantage ($P < 0.01$). The authors attribute the increase to the addition of glucose. Simultaneously the 50 percent egg yolk level of the control was reduced to about 17 percent egg yolk by volume in the yolk-citrate-glucose extender, and this change may also have contributed to the fertility level observed. Over a period of years the level of egg yolk used in the citrate-buffered extender devised by Salisbury et al. (4) has gradually been reduced to 20 to 25 percent by volume, and glucose or fructose is sometimes added.

16-3.2c Ratio of Yolk to Buffer. The early work was done with equal volumes of egg yolk and buffer. Using smaller amounts of egg yolk often yielded inferior results, probably because of the mitigating effect of the egg yolk on the hyperosmotic citrate solutions used and on the high concentrations of phosphate ions in the phosphate extender.

Table 16-1 shows a summary of fertility results, which clearly indicate that there was generally no fertility advantage in reducing the proportion of egg yolk and simultaneously increasing the sodium citrate but that, for convenience, the proportion could be reduced considerably. With the development of more complete buffers to combine with egg yolk, however, a reduction in the volume of egg yolk from 50 to 20 percent improved sperm survival with many buffer combinations tested (66). Fertility was also improved significantly over the standard 50 percent yolk-citrate extender (see Table 16-1).

Under most conditions where osmolarity, pH, and other characteristics of the buffer alone appear to be optimal for sperm survival, egg yolk level may be reduced from 20 to 1 percent. This is particularly true at ambient temperatures (see Section 16-4). Table 16-2 shows an example of the importance of the egg yolk level as it interacts with four other factors during an 8-day storage period. The effects of extender, molarity, and yolk level were large ($P < 0.005$), accounting for a majority of the non-bull variance. Temperature had no effect, showing that with proper media bull spermatozoa can survive for 8 days at 25°C as well as at 5°C. Also pH had no effect. By far the largest interactions were extender $\times$ temperature (CUE—Cornell University Extender—was best

Table 16-1. Fe-
proportion of egg

% egg		
50	33	25
67.1		66.0
66.7		63.9
70.8		69.2
73.5		
66.2		
65.8		
63.1		
		74.0
73.4*		

*16-week nonreturns.

^b6-month nonreturns.

^c120-150-day nonreturn

^d12-week nonreturns.

* $P \leq 0.01$ differences

at 25°C and Tris was best egg yolk was clearly best large and significant in $\times$ molarity, and molarity

16-3.2d Complex Citrate developed complex buffer plex buffers, and they reported on the room temperature, but it well. Table 16-3 shows the CU-16, and of two modifications (77). Modifications of the 0.3 to 1.2 percent and these and similar extenders gassed with CO₂. The latter two modified IVT extenders age 60-90-day nonreturn yolk-skim-milk control.

Table 16-1. Fertility results (percent nonreturns) as the proportion of egg yolk to citrate is decreased.

% egg yolk						Total inseminations	Ref.
50	33	25	20	17	12.5		
67.1		66.0				3,172	(69) ^a
66.7		63.9				2,929	(70) ^b
70.8		69.2				2,591	(71) ^c
73.5			72.3			11,013	(72) ^d
66.2			69.5			5,908	(73)
65.8				63.4		2,809	(70) ^b
63.1					62.4	2,484	(70) ^b
		74.0 ^e		66.2 ^e		2,713	(71) ^c
73.4 ^e			76.3 ^e			18,494	(74) ^e

^a16-week nonreturns.^b6-month nonreturns.^c120-150-day nonreturns.^d12-week nonreturns.^e $P \leq 0.01$ differences due to chance.

at 25°C and Tris was best at 5°C), and temperature $\times$ level of yolk (20 percent egg yolk was clearly best at 5°C and 5 percent yolk was best at 26°C). Other large and significant interactions were extender $\times$ molarity, temperature $\times$ molarity, and molarity $\times$ egg yolk level.

16-3.2d Complex Citrate-Buffered Yolk Extenders. Foote et al. (74, 75) developed complex buffered yolk extenders based on previous work with complex buffers, and they reported beneficial effects of glycine (76) and CO₂ (24). They also reported on the use of Illini Variable Temperature (IVT) extender at room temperature, but it has been used as a bull-semen extender at 5°C as well. Table 16-3 shows the original composition of this extender, of CUE and CU-16, and of two modifications called 14 and 14G developed in New Zealand (77). Modifications of the IVT have been made by increasing the glucose from 0.3 to 1.2 percent and the egg yolk from 10 to 20 percent. The composition of these and similar extenders is given by Bartlett and VanDemark (25). All are gassed with CO₂. The latter workers, in a limited test totaling 765 cows, found that two modified IVT extenders containing 20 percent egg yolk yielded average 60-90-day nonreturns over a 5-day use period comparable to a citrate-yolk-skim-milk control.

An extensive test (78) of a similar CO₂-gassed extender with glycine and glutathione added gave results as follows:

Age of semen (days)	CO ₂ extender		Yolk-citrate	
	Cows bred	% NR	Cows bred	% NR
1	724	78	279	74
2	2,293	76	646	68
3	2,272	72	506	62
4	2,005	66	50	60
5-7	1,363	64	—	—

Melrose (79) obtained a 56 percent 112-day NR rate for bull sperm in IVT used at 5°C over a period of 2 to 6 days. A 9 percent skim-milk powder-glycerol control averaged only 40 percent over this period. It is clear from these examples that semen in IVT can be used successfully for several days at 5°C. One disadvantage has been the variability in results achieved, which presumably reflects varying degrees of maintaining the proper CO₂ level following gassing (26).

The CUE extender (74, 75) was developed to maintain proper pH and to generate CO₂ through the use of bicarbonate-citric acid buffer system. This was compared with a simpler extender, CU-16 (see Table 16-3). When bull semen was extended to 10 million motile spermatozoa per milliliter and stored for 12 days at 5°C, sperm survival in CUE and CU-16 was significantly better than in the yolk-citrate control. A field trial with semen processed as in the motility trial, but with most semen used for insemination within three days of collection, gave the following results (75):

Extender	No. 1st inseminations	% 60-90-day NR
2.9% citrate (EYC)	6,069	73.4
CUE	6,280	76.6
CU-16	6,145	76.0

When put into routine use in the field 290,053 inseminations averaged 74.9 percent 60-90-day NR, an increase of two percentage units over a comparable period when the 2.9 percent citrate-yolk extender was used. Singleton and Josey (80) also reported that CUE and CU-16 were superior to EYC for bull sperm at 5°C.

Table 16
buffer ty
ejaculate

Buffer concentration	pH
50% ^b	6.5 6.7
75%	6.5 6.7
100%	6.5 6.7
0.15M	6.5 6.7
0.20M	6.5 6.7
0.25M	6.5 6.7

*Foote, R. H. U
^bBuffer concentr.

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Table 16-2. Sperm motility as affected by storage temperature, buffer type, osmolarity, pH, and egg yolk levels. Means of fifteen ejaculates after storage for eight days.^a

Buffer concentration	pH	Stored at 5°C				Stored at ambient temperature (25°C)			
		% egg yolk				% egg yolk			
		0	1	5	20	0	1	5	20
(CUE extender)									
50% ^b	6.50	11	27	27	43	20	20	31	38
	6.75	17	33	28	39	17	29	28	43
75%	6.50	1	54	49	51	50	57	48	45
	6.75	0	53	49	58	31	54	51	43
100%	6.50	0	47	46	46	42	55	55	41
	6.75	0	47	47	49	33	59	54	50
(Tris-yolk extender)									
0.15M	6.50	5	30	37	49	13	13	14	14
	6.75	3	31	30	50	7	12	5	15
0.20M	6.50	3	34	41	44	18	15	29	33
	6.75	2	27	43	53	15	17	25	23
0.25M	6.50	0	28	31	29	16	23	34	27
	6.75	0	35	43	43	13	25	31	16

^aFoots. R. H. Unpublished data. Cornell University. 1960.

^bBuffer concentration as a percentage of strength shown in Table 16-3.

Shannon (77) reported on extensive field testing of bull semen (see Table 16-4) with several of the extenders listed in Table 16-3. All extenders contained 20 percent egg yolk by volume. With the short breeding season in New Zealand, fertility is calculated on the basis of 49-day NR to insemination. The fertility results were significantly lower in the control yolk-citrate extender, which confirmed previous studies (75). Particularly striking was the difference in fertility after two days' storage. It is believed that the ability of the CUE and CUE-G extenders to maintain high fertility over a period of several days is due to the CO₂ liberated from the sodium bicarbonate. However, part of the beneficial effect observed previously with extenders 14, 14G, and CU-16 appears to result from an improvement in the nonelectrolyte balance through the addition of glucose and glycine.

Table 16-3. Composition of buffered yolk extenders.^a

Component	Citrate	g/100 ml of distilled water				
		IVT ^b	CUE	CU-16	14	14G
Sodium citrate dihydrate	2.9	2.00	1.45	1.45	2.00	2.00
Sodium bicarbonate	—	.21	.21	—	—	—
Potassium chloride	—	.04	.04	—	—	—
Citric acid monohydrate	—	—	.09	—	—	—
Glucose	—	.30	.30	1.25	.30	.30
Glycine	—	—	.94	.94	1.00	1.00
Glycerol	—	—	—	—	—	1.25
Sulfanilamide	0.6	.30	.30	.30	—	—

^aFinal extenders consisted of 20 parts egg yolk and 80 parts buffer to which antibiotics were added. In some studies 50% egg yolk was combined with the citrate. Adapted from references 24, 74, 75, and 77.

^bGassed with CO₂.

Table 16-4. Fertility of bull spermatozoa stored at 5°C in yolk extenders, based on 49-day nonreturns (NR)^a.

Extender ^b	Age of semen when used					
	Day of collection		Stored 1 day		Stored 2 days	
	No. inseminations	% NR	No. inseminations	% NR	No. inseminations	% NR
CUE	59,091	62.8	25,309	65.2	1,190	62.4
CUE-G	30,058	63.1	13,909	64.9	2,392	62.9
14	73,993	62.8	37,758	63.5	2,681	59.2
14G	35,351	62.7	17,597	64.9	932	60.0
Citrate	98,605	60.6 ^b	44,610	60.8 ^c	2,788	51.5 ^c

^aFrom Shannon. *New Zealand J. Agric. Res.* 7:357-363. 1964.

^bSee Table 16-3 for composition. CUE-G was CUE plus 1.25% glycerol (w/v). All extenders contained 20% egg yolk by volume.

^cSignificantly different from all others, $P < 0.01$.

The CUE formula without appreciable modifications follow.

Extender
CUE
CUE + catalase, 2%
CUE
CUE + catalase, 2%
CUE
CUE + catalase, 1%
CUE, pH 6.80 (control)
CUE, pH 6.65
CUE, pH 6.50
CUE, 300 mosmol
CUE, 333 mosmol

^aNone of the differences

Catalase has been shown to lead to hydrogen peroxide in egg yolk, however, dark storage conditions.

The pH of CUE can be affected by increasing with CUE at the level of control CUE. Pickers poorer fertility with

Other studies (critical). These and have yielded high few days at 5°C.

New Zealand Table 16-3) by gas extender called "C dihydrate, 0.3 percent caproic acid percent egg yolk, fertility results were

The CUE formula has been modified slightly by different workers but without appreciably changing fertility results. Several examples of these modifications follow.

Extender	No. 1st inseminations	% 60-90-day NR ^a	Reference
CUE	6,913	71.0	(45)
CUE + catalase, 20 µg/ml	7,615	71.5	
CUE	10,309	76.1	(45)
CUE + catalase, 20 µg/ml	10,951	74.6	
CUE	6,064	72.9	(81)
CUE + catalase, 1 µg/ml	6,243	73.2	
CUE, pH 6.80 (control)	23,727	74.5	(23)
CUE, pH 6.65	24,779	74.7	
CUE, pH 6.50	22,577	74.7	
CUE, 300 mosmols	12,797	74.0	(15)
CUE, 333 mosmols	13,966	74.0	

^aNone of the differences was statistically significant, $P > 0.05$.

Catalase has been shown to protect sperm under conditions that presumably lead to hydrogen peroxide formation (33, 41). The small amount of catalase in egg yolk, however, is apparently sufficient to protect sperm under the usual dark storage conditions at 5°C, where added catalase has only minor effects.

The pH of CUE extender may be decreased to at least 6.5 without harmful effect by increasing the citric acid component of the buffer. Other experiments with CUE at the lower pH have given actual fertility values higher than for control CUE. Pickett et al. (82) recommended the lower pH range and obtained poorer fertility with CUE as the pH approached 7.0.

Other studies (15) indicate that minor changes in osmotic pressure are not critical. These and numerous other trials with CUE or modifications (83-86) have yielded high fertility results with the extenders used over a period of a few days at 5°C.

New Zealand researchers (87) improved the citrate-buffered yolk (see Table 16-3) by gassing with nitrogen and adding caproic acid. This led to an extender called "Caprogen." Caprogen consists of 2.0 percent sodium citrate dihydrate, 0.3 percent glucose, 1.0 percent glycine, 1.25 percent glycerol, 0.0325 percent caproic acid, and 0.01 percent sulfacetamide, to which is added 20 percent egg yolk, penicillin, and streptomycin. In 1961 (88) the following fertility results were reported.

Age of semen	14G		14G + caproic acid	
	No. inseminations	% 49-day NR	No. inseminations	% 49-day NR
Day of collection	224,000	64.1	42,000	63.7
Day after collection	96,000	64.3	17,000	66.9

The addition of caproic acid increased NR rates on semen stored for a day at 5°C ($P < 0.01$). A subsequent trial was initiated comparing caproic acid additions and nitrogen gassing. These results are summarized in Table 16-5. The caproic acid and/or gassing with nitrogen improved fertility of semen used after the first day. Thereafter Caprogen, called 14GCN, was used routinely in New Zealand for extending sperm stored at 5°C. Later (see Section 16-4) this was modified for ambient temperature use by reducing the egg yolk content. Balasov et al. (89) also reported that caproic acid was beneficial when added to a yolk-citrate-glucose extender.

Following the work of Roy and Bishop (76) there were numerous reports that yolk-glycine and yolk-glycine-glucose improved sperm survival (5, 57). However, fertility results were often disappointing. For example, Graham and Erickson (90) used a yolk-glucose-glycine extender to inseminate 3,982 cows and obtained 65.8 and 35.7 percent 60–90-day NR on the day of collection and the day after collection. These results were 7.6 and 24 percent below the heated

Table 16-5. Fertility (% 49-day nonreturns) of bull spermatozoa stored at 5°C in extenders gassed with nitrogen and/or containing caproic acid.^a

Extender ^b	Age of semen when used					
	Day of collection		Stored 1 day		Stored 2 days	
	No. inseminations	% NR	No. inseminations	% NR	No. inseminations	% NR
14G (Control)	101,982	65.2	56,687	65.4 ^c	2,061	62.3
14GC	99,420	65.6	52,064	66.7 ^d	5,854	63.5
14GN	50,637	65.8	27,163	66.2 ^d	2,359	61.9
14GCN	52,436	65.8	35,435	67.6 ^d	739	64.8

^aFrom New Zealand Dairy Board. 48th Farm Production Rpt. 1971–1972.

^bC = caproic acid; N = nitrogen gassing; CN combination is called Caprogen.

^{c,d}Numbers with different superscripts in the same column, $P < 0.01$.

whole milk containing frequently, glycine h such as CUE or

A variety of literature, but th been summarized to fertility was sium-phosphate 15°C, 74.3 percent in a yolk-citrate resulted in 65.0

16-3.2e Tris-But buffers of Good (as extenders for et al. (92) to be vided excellent (18, 19) bull sp superior to that was obtained in 6.75. A nomogr pH characteris

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Only Tris Extensive studi timum levels o 18). Glycerol w ficial effects of pound in exte 16-6) showed avoiding the ti with other ext but motility of and when the

whole milk control used simultaneously to inseminate 3,722 cows. Consequently, glycine has been little used recently, except in more complex extenders such as CUE or Caprogen.

A variety of buffered yolk preparations have been reported in the Russian literature, but the exact composition is often not given. These reports have been summarized by Turton (91). One extender extensively tested with regard to fertility was a yolk-sodium-citrate-saccharose-sodium-bicarbonate-potassium-phosphate extender. When semen was stored for up to three days at 5 to 15°C, 74.3 percent of 5,998 cows inseminated conceived. Semen stored at 0°C in a yolk-citrate-glucose control extender and used to inseminate 6,231 cows resulted in 65.0 percent conception.

16-3.2e Tris-Buffered Egg Yolk Extenders The development of zwitter-ion buffers of Good (92) has led to the examination of many for buffering egg yolk as extenders for bull spermatozoa (18-22). These buffers were shown by Good et al. (92) to be relatively harmless to various cell suspensions, and they provided excellent buffering capacity over a suitable pH range. In early studies (18, 19) bull sperm survival at 5°C in Tris-buffered egg yolk was equal or superior to that found for egg yolk citrate and CUE. Optimum sperm survival was obtained in 0.2 molar Tris-yolk extender, adjusted with citric acid to pH 6.75. A nomogram for preparing Tris solutions with different osmolarity and pH characteristics has been published (14).

Among the compounds that have been tested (21) using sperm motility as a criterion of suitability, are TRIS, tris (hydroxymethyl) aminomethane; TES, N-tris (hydroxymethyl) methyl-2-aminomethanesulphonic acid; MES, 2-(N-morpholino) ethanesulphonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; PIPES, piperazine-N,N'-bis(2 ethane sulfonic acid); MOPS, 2-(N-morpholino) propane sulfonic acid; and BES, N, N bis (2-hydroxyethyl)-2 aminoethane sulfonic acid. These compounds can be combined and titrated to provide buffers ranging in pH and osmolarity that are relatively innocuous to spermatozoa (20). They can be combined with egg yolk to give suitable extenders for temperatures ranging from 25 to -196°C.

Only Tris has been extensively fertility-tested with bull spermatozoa. Extensive studies were carried out before the fertility testing to establish optimum levels of Tris in combination with egg yolk and other substances (14, 18). Glycerol was included as one component because of the observed beneficial effects of glycerol at 5°C in milk extenders and the need for such a compound in extenders for freezing semen. The results of one study (see Table 16-6) showed that glycerol could be included in the initial extender, thus avoiding the time-consuming steps of adding glycerol at 5°C, as is often done with other extenders. There was no difference in times of glycerol additions, but motility of spermatozoa was lower in media with a pH of 6.25 ($P < 0.01$) and when the molarity of the Tris was 0.25 ($P < 0.01$).

Table 16-6. Spermatozoan motility when glycerol was added directly to Tris-yolk extenders or after cooling to 5°C.^a (Average of 20 samples stored for 8 days at 5°C.)

Glycerol added	Molarity of Tris	pH			
		6.25	6.50	6.75	
(% motile spermatozoa)					
Initially	.20	47	55	55	
	.25	40	49	52	
At 5°C	.20	47	55	53	
	.25	42	47	51	

^aFrom Foote. *J. Dairy Sci.* 53:1475-1477. 1970.

The Tris extender that was selected for fertility testing (15) consisted of the following:

Tris	3.028 g
Citric acid monohydrate	1.675 g
Fructose	1.25 g
Glycerol	8.0 ml
Glass redistilled water	92.0 ml
Egg yolk (+ antibiotics)	25.0 ml

Semen was extended an average of 1:108 to give 8 million motile sperm per milliliter and the 0.2M Tris-fructose-glycerol-yolk extender, pH 6.75, was compared with CUE.

Extender	No. 1st inseminations	% 60-90-day NR
CUE	5,981	73.0
Tris	5,673	73.3

These results showed clearly that most of the ions in traditional buffers could be replaced by Tris. Other field trials established that glycerol was beneficial in the Tris extender (see Table 16-7). The difference, in favor of glycerol, was 2.5 percent ($P < 0.01$). There was no statistically significant effect of pH, although the lower pH tends consistently to be associated with higher fertility (23). Tris and CUE gave equal fertility results. Another field trial with over

40,000 inseminations resulted in 73.4 percent for Tris.

Tris has been used for CUE (85) 4,080 inseminations with 69.7 percent for Tris (86) the CUE-Tris extender over a period of five weeks for use in the percent 60-90-day extender is 7.57 g grams sodium citrate, 7.57 g grams glucose, 7.57 g added 10 to 20 percent.

It is clear that the solutions that can be used for ambulatory

16-3.2f Other Experiments et al. (94). Preparation of the chazalae remains citrate/whole-egg sulfanilamide in sodium with sulfanilamide survival. The whole returns compared have not been a

Table 16-7
vs CUE.^a

Treatments
CUE Control
Tris, pH 6.5
Tris, pH 6.75
Tris-gly, pH 6.5
Tris-gly, pH 6.75

^aFrom Foote. .

40,000 inseminations resulted in 73.0 percent 60-90-day NR for CUE and 73.4 percent for Tris, thus confirming the equivalency of the two extenders.

Tris has been used successfully for storing buffalo spermatozoa (93). It has been used for titrating other zwitter-ion buffers (21). When combined with CUE (85) 4,080 inseminations resulted in 72.3 percent 60-90-day NR versus 69.7 percent for Spermasol T. When compared with Spermasol milk extender (86) the CUE-Tris was superior, declining only two percentage units in fertility over a period of four days. When semen was collected and processed only twice a week for use throughout the week, 137,074 first inseminations gave 70.2 percent 60-90-day NR. The formula for preparing one liter of the CUE-Tris extender is 7.57 grams Tris, 2.50 grams fructose, 5.53 grams citric acid, 10.88 grams sodium citrate, 1.35 grams sodium bicarbonate, 0.25 gram KCl, 2.25 grams glucose, 7.03 percent glycerol, and 2.25 grams sulfanilamide. To this is added 10 to 20 percent egg yolk and antibiotics.

It is clear that Tris types of organic buffers provide a versatile series of solutions that can be used to formulate desirable semen extenders. They are suitable for ambient temperature storage, refrigerated, or frozen semen.

16-3.2f Other Egg Extenders. A whole-egg extender was tested by Dunn et al. (94). Preparation of the extender was simple because the whole eggs with the chalazae removed were simply blended and added to buffer. The pH of a citrate/whole-egg medium was adjusted from about 7.9 to 6.7 by replacing sulfanilamide in the conventional 2.9 percent citrate-sulfanilamide-yolk medium with sulfasuxidine. Adjustment of the pH is essential for good sperm survival. The whole-egg formulation averaged 61.6 percent 60-90-day non-returns compared to 62.3 percent for the egg yolk control. Whole-egg extenders have not been adopted commercially.

Table 16-7. Fertility of bull spermatozoa in liquid Tris extender vs CUE.^a

Treatments	Osmolarity (mosmols)	No. 1st services	% 60-90-day NR
CUE Control	333	13,966	74.0
Tris, pH 6.5	323	14,376	71.9
Tris, pH 6.75	317	13,933	71.2
Tris-gly, pH 6.5	—	13,630	74.0
Tris-gly, pH 6.75	—	12,903	73.1

^aFrom Foote, J. *Dairy Sci.* 53:1475-1477, 1970.

Various prepared extenders incorporating egg yolk have been marketed commercially (5, 41, 95). In Japan Neoseminan is an ampuled sterilized product reported to be stable for several years. It consists of egg yolk, sodium citrate, potassium citrate, sodium phosphate, glucose, homosulfamine, sodium sulfamesadin, and chlorpromazine and is gassed with CO₂. Spermasol (see Section 16-3.3e) has been used principally in Germany. Many formulations containing sodium citrate, potassium phosphate, and gelatin have been combined with either egg yolk or milk. Other extender formulations have been marketed as well (60). Although they offer convenience, any of the complete formulations containing egg yolk or similar organic material probably have limited shelf life. Large AI organizations therefore prepare their own extenders shortly before use.

16-3.3a Milk—History and Heating. Phillips and Lardy (96) observed that evaporated milk could maintain sperm for only 48 hours. Fresh semen gave higher fertility than semen extended and stored in yolk-phosphate or in autoclaved milk (97). Better results were obtained by Michajilov (98), who used boiled filtered milk for jack semen. A canned sterile skim milk was easy to prepare, sperm were clearly visible, and fertility was superior to that allowed by the yolk-citrate used previously (99). Later Jacquet and Cassou (100) found that the quality of the canned milk product varied and they recommended that 10 percent by weight of powdered milk plus antibiotics be added to double-distilled water and that egg yolk also be included up to 10 percent of the final volume.

Milk preparations were being investigated simultaneously by Almquist and associates, and details of proper milk handling as a bull semen extender were described (101). Milk must be heated beyond the pasteurization point to destroy lactenin, a spermicide (102). Sulfhydryl groups released by heating probably inactivate the toxic factor. Recommended procedure is to heat milk in a double boiler to 92 to 95°C for 10 minutes, cool it to 5°C, and add antibiotics. The extender is then ready for use. Other methods of inhibiting the toxic factor have been reviewed (5, 41); they include the use of thioglycolic acid or cysteine. Both contain sulfhydryl groups. Jones (103) postulated that excess cysteine might be harmful and later reported that dialysis to remove excess cysteine was beneficial (104). Heating, however, was preferable. Many laboratories in the 1950s observed that egg yolk also made heating the milk unnecessary and this was documented in the literature (105).

Fortified skim milk was found, as a semen extender, to be detrimental to spermatozoa (106). Different batches of skim milk also required different degrees of heating to inactivate the toxic factor (107). Too much heat can be harmful, possibly because of its effect on lactose (103). Others have observed variability in batches of liquid milk and dried milk. This and the different procedures used in heat treatment of milk may be responsible for some of the

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variability in the fertility results that investigators have obtained in comparing milk and egg yolk extenders.

16-3.3b Liquid and Dried Milk Preparations. Liquid whole milk, skim milk, cream, and various reconstituted powdered milks have been tested as bull semen extenders. The early literature has been reviewed (5, 41). Since sperm motility is difficult to assess in many milk preparations only the fertility results of studies are meaningful.

Table 16-8 summarizes fertility tests run on several milk extenders in which antibiotics were used. Most of the fertility results were based on 60-90-day nonreturns to service, but if fertility was based on other time intervals the comparisons within an experiment were made on the same basis. The first series of experiments all included egg yolk-citrate as a control. In general egg yolk-citrate and milk extenders gave comparable results. Most differences were small and appeared to be due to chance. Variations in the levels of egg yolk (20 to 50 percent), citrate (2.9 to 3.6 percent), and antibiotics, as well as the formulation and batches of milk used, also presumably contribute to the variations observed. Saacke et al. (106) called attention to the importance of milk solids and osmolarity: Although milk proteins can protect spermatozoa against moderate shifts in osmolarity (104) failure to observe the principles of sperm preservation discussed earlier in this chapter can reduce fertility.

In the studies by Almquist (109) and Melrose et al. (114) semen from bulls exhibiting low fertility appeared to benefit from being stored in milk. It is possible that the antibiotics were somehow more effective in the milk preparation as processed. Morgan et al. (119) showed that *Vibrio fetus* infection was controlled by two hours' exposure at 5°C to streptomycin in skim milk but not in egg yolk. At the time of these studies vibriosis was widespread in cattle and had not yet been eradicated from most bull studs.

Homogenized milk, homogenized cream (9 percent fat), and skim milk are capable of yielding comparable fertility results. Skim milk, however, allows spermatozoa to be seen microscopically and sperm viability can therefore easily be assessed. Moreover, skim milk powder is readily available and inexpensive. Large batches can be pretested and, if found to be satisfactory, can be used with confidence in their uniformity. The sterilized cream (115) is convenient to use because it requires no additional heating, and large batches can be secured for use over a considerable period of time. Reconstituted buttermilk (not shown in Table 16-8) can also be used (120). Spermatozoa extended in reconstituted buttermilk (10.8 grams with 89.2 milliliters of distilled water plus antibiotics) gave good results in a limited fertility test when compared with yolk-citrate extended semen.

16-3.3c Milk Combined with Sugar, Glycine, and Glycerol. Although the fertility of bull spermatozoa extended in milk was initially good, it often declined rapidly with storage at 5°C. This was also true of the original yolk-citrate

Table 16-8. Fertility of bull spermatozoa in heated fresh or powdered milk with sugars, glycine, or glycerol.

Treatment*	No. 1st inseminations	% NR	Reference
Homogenized milk	2,931	59.9 ^b	(73)
Homogenized milk-sulfanilamide	4,375	64.6	
Yolk-citrate-sulfanilamide	2,892	69.5	
Homogenized milk	1,570	76.1	(108)
Homogenized milk + cornstarch	1,554	74.3	
Yolk-citrate	1,565	75.9	
Homogenized milk + cornstarch	2,852	65.3	(108)
Skim milk + cornstarch	2,873	67.8	
Yolk-citrate-sulfanilamide	2,674	66.8	
Homogenized milk	4,178	71.6	(109)
Yolk-citrate	4,222	64.2 ^b	
Homogenized milk	10,012	69.4	(110)
Yolk-citrate	9,927	71.2	
Homogenized milk	4,637	68.8	(111)
Yolk-citrate	5,051	69.9	
Skim milk	6,850	70.7	
Yolk-citrate	7,979	69.1	
Homogenized or skim milk	23,422	69.8	(112)
Yolk-citrate-sulfanilamide	24,269	69.6	
Skim milk	44,746	69.0	(113)
Skim milk powder, 10% w/v	48,013	69.3	
Skim milk powder, 9% w/v	5,076	68.4	(114)
Yolk-citrate	5,113	63.5 ^b	
Skim milk powder, 9% w/v	2,276	65.7	(114)
Skim milk powder + 12.5% yolk	2,339	68.1	
Skim milk powder, 9% w/v	1,521	66.3	(114)
Skim milk powder + 3% glycine	1,421	67.2	
Skim milk powder, 9% w/v	2,025	67.3	(114)
Skim milk (liquid)	1,949	68.9	
Skim milk	2,263	69.2	(115)
Sterilized homogenized cream	2,931	69.4	
Yolk-citrate	2,370	63.6	
Homogenized milk	25,599	71.3	(116)
Homogenized milk + 1.25% fructose	25,282	72.4	
Skim milk	14,319	61.5	(117)
Skim milk + 10% glycerol		64.5	
Skim or homogenized milk	10,845	63.7	(118)
Skim or homogenized milk + 10% glycerol	10,831	71.5 ^b	

*All extenders contained antibiotics (streptomycin and often penicillin) and all liquid milks were heated excepting when egg yolk was added. Egg yolk content varied from 20 to 50% in the yolk-citrate controls.

^bValues that differ significantly from others within an experiment, $P < 0.05$.

extender, and yolk extender: hexoses, although fat-decomposition utilize lactose glucose anaerobically homogenized and the decline in fertility for spermatozoa. The effect of Foote (73) on day NR in his scale experiment added to heuristics and 3 days later (118) also showed that stored for several days found that the last two days follows:

Age

No dilution extended was been added liveability, and induced at 5°C was 64.6 to 55.5% skim milk indicate the initial fertility several days

extender, and was partially responsible for the development of more complex yolk extenders created to prolong fertility of unfrozen semen. Milk is low in hexoses, although traces of glucose are present, and it contains lecithin and the fat-decomposition products glycerol and organic acids. Spermatozoa cannot utilize lactose, but they can metabolize the other substances aerobically and glucose anaerobically. No advantage was found in adding fructose to heated homogenized milk (see Table 16-8). Semen was used uniformly for four days and the decline in fertility with fructose added was 5.5 percent versus 4.6 percent for the milk extender alone (116). Thus at 5°C an additional energy source for spermatozoa appeared unnecessary.

The effect of adding glycerol to milk is usually favorable. Bratton and Foote (73) obtained, with and without glycerol, 65.8 and 64.4 percent 60-90-day NR in heated homogenized milk. Williams et al. (121) conducted a large scale experiment (14,341 inseminations) and reported that 10 percent glycerol added to heated homogenized milk increased the 60-90-day NR after 1, 2, and 3 days of storage at 4°C by 2.5, 3.5, and 4.2 percent ($P < 0.01$). Almquist (118) also showed that glycerol markedly improved the fertility of bull sperm stored for several days at 5°C. On the other hand, O'Connor and Smith (117) found that glycerol helped to maintain the fertility of bull sperm only during the last two of four days of storage at 5°C. A comparison of these results follows:

Age of semen	No. 1st inseminations		Advantage of glycerol in % NR	
	O'Connor	Almquist	O'Connor	Almquist
1 day	3,682	7,106	- 3.1	+ 2.3
2 days	3,494	8,707	- 1.3	+ 7.4
3 days	3,688	3,405	+ 4.7	+ 12.2
4 days	3,455	2,458	+ 10.6	+ 19.5

No difference in fertility was found between semen from the same source extended with heated skim milk to which either 5 or 10 percent glycerol had been added (122), although 5 percent glycerol gave the best spermatozoan liveability. Glycerol added to skim milk at room temperature reduced motility and induced morphological changes in spermatozoa. Slow addition of glycerol at 5°C was recommended. In another study (79) fertility was reduced from 64.6 to 55.6 percent by including 10 percent glycerol initially in a 9 percent skim milk powder extender mixed with sperm cells at 20°C. Overall, the studies indicate that, with the proper preparation of milk and milk-glycerol extenders, initial fertility of spermatozoa in each is similar, but is better maintained for several days at 5°C with milk-glycerol extender.

16-3.3d Milk-Egg Yolk Combinations. After early work indicated that small volumes of egg yolk added to milk might improve sperm survival and inactivate the spermicide in milk, many combinations were tested. The early work was reviewed in the previous edition of this text (123). Table 16-9 shows several combinations of milk with yolk and glycine or glycerol. All liquid milks and most reconstituted milks used were heated to 92°C for 10 minutes, and all extenders listed in the table contained antibiotics.

The addition of glycine to milk was based on the sometimes favorable results obtained with yolk-glycine extenders (see previous sections). However, it is clear that under the conditions tested glycine did not improve fertility; in fact, when it was combined with glycerol and milk-yolk (see Table 16-9) it decidedly depressed fertility. Hence glycine has not been used as a regular constituent of milk extenders.

Table 16-9. Fertility of bull spermatozoa in milk-yolk combinations.

Treatment	No. 1st inseminations	% NR	Reference
Homogenized milk	3,033	73.7	(124)
50% milk-25% yolk- 25% of a 3% glycine solution	2,848	72.3	
Homogenized milk + 10% glycerol	4,850	71.4	(124)
Milk-yolk-glycine + 10% glycerol	4,078	60.9 ^b	
Skim milk-10% yolk + 8% glycerol	2,552	73.5	(125)
Yolk-citrate + 6.32% glycerol	2,542	74.4	
Skim milk + 5% yolk ^a	4,045	67.0	(126)
Skim milk-5% yolk + 10% glycerol	4,032	67.0	
Skim milk-5% yolk	8,438	76.3	(126)
Skim milk-5% yolk + 10% glycerol	7,947	77.7	
Milk-yolk	3,665	63.4 ^b	(127)
CU-16 (yolk base)	3,853	68.3	
9% skim milk powder, 12.5% yolk	11,524	63.3	(79)
CUE (complex buffered yolk)	11,339	64.6	
9% skim milk powder, 6% yolk	20,610	63.2	(128)
9% skim milk powder, 6% yolk, 1.5% glycerol	20,630	63.8	
9% skim milk powder, 6% yolk	16,983	63.3	(128)
9% skim milk powder, 10% yolk, 3% glycerol	16,983	62.6	

^aIn the first study by these authors glycerol was added at room temperature and 90-120-day % NR calculated. In the second study glycerol was added at 5°C and 30-60-day % NR calculated.

^bP < 0.01 comparing fertility within experiments.

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The addition of glycerol to liquid milk and egg yolk or to powdered milk with egg yolk was consistently without measurable effects. Glycerol does not appear to improve the fertility of semen stored for several days in milk-yolk extenders, in contrast with its demonstrated effect when added to milk alone (128). It is possible that egg yolk serves a similar function to that of glycerol in milk extenders, but the results of appropriate tests of that hypothesis, using the milk and milk-yolk extenders with and without glycerol, have not proved the hypothesis. It appears, however, that semen can be added directly to glycerolized milk-yolk extender (126). This could simplify processing, especially of frozen semen.

Milk-yolk extender seems comparable to CUE (79), although after 3 days of storage the 112-day nonreturn rates were 62.5 percent for CUE and 57.8 percent for milk-yolk extender. A report by Hutchinson and Cooper (129) indicated that 3 percent glycerol added to 87 parts of heated skim milk and 10 parts of egg yolk improved fertility substantially over a yolk-citrate control. A similar extender made from powdered skim milk (128) was not superior to milk-yolk when bull spermatozoa were stored for up to 3 days before insemination.

Thus the simplest satisfactory milk-yolk semen extender appears to be one made of either heated fresh or powdered skim milk (9 to 10 percent solids) with 5 to 10 percent fresh egg yolk added. Such an extender was used regularly by several AI organizations until the practice of freezing semen came into use.

16-3.3e Other Extenders. Various commercial preparations have been used successfully. Spermasol, for example, is an egg yolk-citrate-phosphate-gelatin product that can be combined with milk (130); Laiciphos is a powdered milk extender that gives good fertility when mixed with 10 percent fresh egg yolk (131). Tomato juice and other vegetable materials have been combined with yolk (41) and countless variations in recipes for semen extenders can be found in the literature (5, 41, 132). Examples of those tested extensively for ability to yield high fertility have been summarized in the tables. Certain extenders designed primarily for storage at ambient temperatures (see Section 16.4) have received limited use at 5°C.

Testing extenders for commercial insemination of cattle has served as a basis for developing extenders for other species. Similar extenders preserve buffalo sperm equally well (133, 134, 135).

16-4 EXTENDERS FOR STORAGE AT AMBIENT TEMPERATURES

16-4.1 Development of Room Temperature Extenders. It is clear from the survival rates of sperm in the epididymis and the female reproductive tract that sperm cells can remain viable at body temperature for significant periods of time. Refrigerating semen at 5°C and freezing semen have greatly increased

its *in vitro* storage life. The need for unrefrigerated semen has therefore diminished, but in some areas refrigeration is not widely available or is expensive. Hence consideration will be given to techniques of preserving bull sperm at ambient temperatures.

Storing spermatozoa in nutrient media at ambient temperatures obviously makes it necessary to control bacteria. According to Foote and Bratton, a combination of sulfanilamide, penicillin, streptomycin, and polymyxin is extremely effective in preventing bacterial growth in bull semen extended with yolk-citrate and stored at 25°C (35). Under these conditions sperm survival is greatly improved and egg yolk is stabilized. Foote and Bratton conclude that "this combination of antibacterial agents gives promise of making possible the development of an extender for bovine semen which will not require refrigeration."

16-4.2 CO₂ Ambient Temperature Extenders. Biochemical control of spermatozoan metabolism is prerequisite for a good extender that can be used at room temperature. Previous sections of this chapter have referred to the Illinois work showing that CO₂ markedly reduced sperm metabolism. When the CO₂ level was properly controlled the motile and fertile life of bull sperm was prolonged (25). A successful ambient temperature extender called IVT (see Table 16-3) was developed (24). These workers reported little change in fertility when a limited number of cows were inseminated with semen stored up to seven days at ambient temperature.

IVT has been used extensively for boar semen where storage temperatures of 15°C or higher are preferable to 5°C. It has not been widely used for bull sperm, however, due to the requirements for CO₂ gassing and for sealing the storage vial so it will hold proper CO₂ levels.

CUE, a related extender (see Section 16-3) that includes glycine and does not require external gassing with CO₂, has given consistently good sperm survival at 25°C. CU-16, a simplified extender, similarly preserves sperm motility. In each of these glycine may promote the desirable production of CO₂ as a result of sperm metabolism. Boye (136) studied temperatures of 5, 10, 15, 20, 25, and 30°C for storing bull spermatozoa in CUE and found 20°C to be the optimum. The insemination of 1,978 cows with CUE-extended semen stored for 1, 2, and 3 days gave 81, 79, and 79 percent NR on a 30-60-day basis.

Abdu (137) reported that ethylenediamine tetracetic acid (EDTA) added to a yolk-citrate-glucose medium gave good fertility when semen was stored in it at 18 to 25°C and used after 3 to 6 days. Only limited breedings were carried out.

16-4.3 Caprogen. By far the most extensive testing of an ambient temperature extender has been done on Caprogen in New Zealand. The initial development of this extender for 5°C storage was given in Section 16-3 (see Table

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16-5). Several pilot experiments indicated that semen could also be handled under the moderate ambient temperatures prevailing during the short breeding season in New Zealand. An extensive test (87) gave the following 49-day NR:

Age of semen	5°C		Ambient temperature	
	No. cows	% NR	No. cows	% NR
Day collected	268,201	65.3	44,354	66.2
Day later	172,663	66.6	29,772	68.9

Because of the good fertility achieved with semen used at ambient temperature, particularly after one day of storage ($P < 0.05$), the majority of inseminations in New Zealand since 1964 have been made with semen kept at this temperature. The optimum temperature range for Caprogen is considered to be 18 to 24°C.

Egg yolk level in Caprogen has been reduced experimentally (87, 138) to as little as 2 percent by volume (see Table 16-10). Five percent egg yolk is used routinely, except for the initial premix with semen, which contains 20 percent yolk. This is consistent with other work (see Table 16-2) showing that less egg yolk is needed with higher temperatures. Furthermore, there appears to be an interaction between spermatozoa and egg yolk levels (87). With low egg yolk levels sperm numbers per insemination can be minimized. The practice for several years in New Zealand has been to extend semen to 4 or 5×10^6 total sperm per milliliter and to use 0.5 milliliter of extended semen per insemination. Earlier work in New Zealand and elsewhere (139)

Table 16-10. Fertility of spermatozoa with different levels of egg yolk in Caprogen extender at ambient temperature.

Total no. inseminations	% egg yolk				Reference
	20	10	5	2	
	(% 49-day NR)				
9,778	67.7	65.7	68.4	—	(87)
425,456	62.8	—	66.1 ^a	—	(87)
16,790	—	—	64.1	66.1 ^b	(87)
12,512	—	—	66.9	68.2 ^b	(87)

^aTreatments differ, $P < 0.01$.

^bTreatments differ, $P < 0.05$.

established that equal numbers of sperm inseminated in 0.5 and 1.0 milliliter of extended semen resulted in similar fertility.

Caprogen contains catalase to decompose the H_2O_2 that is formed as a product of sperm metabolism. This addition consistently and significantly increased fertility by 1 to 2 percent. Examples of results are shown in Section 16-6.

Finally, Caprogen can be used as a liquid extender for resuspending frozen spermatozoa. To accumulate extra semen before the start of the short breeding season New Zealand workers (140) have frozen and accumulated limited numbers of breeding units. The frozen semen is thawed, rediluted with Caprogen, and used at ambient temperature. Fertility of the rediluted semen in three extensive trials was 61.1, 61.1, and 63.3 percent NR. The corresponding regular ambient temperature control results were 62.0, 63.3, and 63.4 percent NR (140). Fertility of the semen collected from individual bulls and then frozen and rediluted was highly correlated with semen collected and used directly. Thus, with proper selection of bulls from which to freeze additional semen, overall fertility would not be appreciably affected.

16-4.4 Coconut Milk. Coconut milk extender (CME) is also used at ambient temperatures. The milk from coconuts had been used as a nutrient medium for the culture of plant embryos and it was combined with other ingredients in an extender for spermatozoa (141). The coconut milk is boiled for 10 minutes and filtered before use. Its formulation, as given by Norman (142), is:

Coconut milk	15 ml
Sodium citrate dihydrate	2.2 g
Sulfanilamide	0.3 g
Egg yolk	5 ml
Distilled water to	100 ml

Included per 100 milliliters of CME are 135 milligrams dihydrostreptomycin sulfate, 10 milligrams polymyxin B sulfate, 1000 units of nystatin and 15,000 units of sterile catalase. For ambient temperatures 0.5 to 1.0 percent egg yolk was suggested (32), but 5 percent egg yolk (v/v) extends the use range of refrigerated semen.

In early fertility tests in the United States CME was found to give results equivalent to skim milk-glycerol and CUE (32). Liveability tests and limited fertility data suggested that spermatozoa might be used for breeding after several days' storage in CME. Semen used after 1 to 5 days in Uganda, Kenya, El Salvador, Paraguay, India, and West Germany gave conception rates ranging from 55 to 72. Malmberg and Israelsson (143) obtained 67.7 percent 84-day NR from 5,760 services with CME used for 4 days at 15 to 25°C, and 65.5

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percent from 5,832 services with frozen-thawed semen. When semen stored in CME was used for several days (about 400 services per day) the respective 84-day NR figures for the seven days were 69, 67, 63, 61, 52, 46, and 39 percent. Fertility dropped markedly after the fourth day. CME can be used with equal success for cattle and buffalo semen (144).

Coconut palm sap was studied (145) as a possible extender that could be prepared in convenient freeze-dried form. It was less effective, however, than the coconut fluids in maintaining sperm motility.

Whether coconut fluids provide any materials unique for sperm preservation is debatable. The analytical grade reagents used in CUE provide a medium as satisfactory as the citrate-coconut milk extender for sperm survival over an 8-day period at 23 to 25°C (146). When CUE with 1 percent egg yolk was used to store 15 samples of bull semen there was no detectable decline in motility for 8 days, compared to a decline from 60 percent motile sperm to 38 percent after 8 days with 20 percent yolk-CUE. This emphasizes again the importance of using a low egg yolk level in ambient temperature extenders. The results from other studies with coconut milk (147) revealed that 5 to 15 percent of skim milk and glucose combined with citrate buffer was equal to CME in maintaining sperm motility at 16 to 27°C.

In summary, extenders that are successful at ambient temperature are generally characterized by a balanced buffer solution, low egg yolk content, catalase, and a combination of antibiotics.

16-5 MICROORGANISMS IN SEMEN AND THEIR CONTROL

Microbial contamination of semen may 1) affect spermatozoa directly, 2) compete for substrate in semen extenders, and 3) infect inseminated females. The result can be lowered conception rates and increased embryonic mortality or abortion. Microorganisms can gain entry into the semen from infected bulls, during semen collection, during semen processing, or during insemination. Thus it is extremely important to use sterile equipment, sterile media, and aseptic procedures at all steps of the program. Obviously, it is important to start with a healthy bull, but this is not always possible, since unknown or undetected diseases can exist in a bull. However, organizations that comply with the American Veterinary Medicine Associations/National Association of Animal Breeder's "Code of Minimum Standards for Health of Bulls and Hygiene of Bull Studs Producing Semen for Artificial Insemination" (148) have effectively controlled known infectious diseases that might be transmissible through semen. Continuous vigilance under proper veterinary supervision is essential. It is regrettable that many custom freezing operations on farms and ranches almost totally ignore the code, thus working to the detriment of AI.

Even under the best conditions some microbial contamination can occur. Chemical treatment of semen to inhibit multiplication of these contaminants

or to kill them without damaging the sperm cells is therefore important. Comprehensive coverage of published reports on the microbiology of bull semen and means for control are available (41, 148-151). The intent of this section is to outline the development and current status of our knowledge in this area. Additional references can be found in the reviews cited.

16-5.1 Microflora of Bull Semen. Since Gunsalus et al. (152) called attention to the numbers of bacteria in semen several reports have appeared (34, 153-157). In general, there are usually fewer than 150,000 organisms per milliliter of semen with a range from no detectable organisms to samples with several millions per milliliter. Cleaning the sheath and underline of the bull (146) and placing a small apron on the chest of the bull and a polyurethane device at the entrance to the artificial vagina (158) effectively reduce contamination at the time of semen collection. Second ejaculates of semen have lower average bacterial counts than first ejaculates.

Bacilli, diphtheroids, micrococci, coliform organisms, streptococci, staphylococci, pseudomonas, actinomyces, protus, yeasts, viral agents, and mycoplasma have all been found in bull semen. Many of the organisms present are not pathogenic and there is therefore no direct correlation between fertility and the total number of organisms present. Most types of organisms, if present in excessive numbers, will reduce sperm survival (153, 156), but this is not always true.

One organism that appears to be associated with low fertility in bulls is *Pseudomonas aeruginosa* (152-154, 157). This organism can be transmitted through frozen semen (159). *Pseudomonas aeruginosa* and *Clostridium pyogenes* occasionally infect the seminal vesicles and then are contributed to semen in the vesicular secretion (150). Other organisms, which are contagious and can infect cows through contaminated semen, include *Brucella abortus*, *Vibrio fetus*, *Trichomonas fetus*, *Leptospira pomona*, *Mycobacterium tuberculosis*, *M. paratuberculosis* (agent of Johne's disease), *Mycoplasma bovine genitalium*, and viral agents that cause infectious bovine rhinotracheitis (IBR) and bovine viral diarrhea (BVD). International shipments of semen must come from areas free from foot and mouth disease and blue tongue, since the viruses responsible for these diseases are transmissible if present in semen.

Efforts are being made to completely eliminate such organisms from bull studs. Through strict programs of testing, selection, isolation, and treatment the major AI organizations have waged a successful battle against most of the organisms listed. Less is known about methods for preventing infection or curing animals with mycoplasma or IBR.

Simultaneously, continuous research is necessary on chemotherapeutic methods of controlling contaminants in semen. Not only can unknown organisms be present, but known organisms, previously sensitive to antibiotics, can

develop resistance of most such organisms survive freezing.

16-5.2 Antibiotic: previous section, numbers of organisms development of ar yolk provided a g were needed that toxic to spermato.

Sulfanilamide. S. pressed the rate of inhibited bacteria sulfanilamide in a percentage units, agreement, part studies indicated after cooling to 5 beneficial effect h one, and the fact idea that sulfanil bition. Sulfanilar and should be e: Other sulfa drug: (162), but none f

Penicillin and Str researched inten. harmless to sper spectrum of mic to 1,000 units of tility of low-ferti rates when 1,000 tomycin per mill had no effect. T Later most of the to streptomycin reports showed t more by addition fertility bulls, an

develop resistance (160). Refrigeration greatly suppresses the multiplication of most such organisms, but does not necessarily stop it (161, 162). Most can survive freezing.

16-5.2 Antibiotics and Other Antimicrobial Agents. As pointed out in the previous section, even when rigid hygienic measures are imposed variable numbers of organisms are present in fresh bull semen. In the early commercial development of artificial insemination semen was stored at 5°C or higher. Egg yolk provided a good nutrient medium for bacterial growth. Effective agents were needed that were easy to use and would control bacterial growth, be non-toxic to spermatozoa, and increase fertility.

Sulfanilamide. Salisbury and coworkers had found that sulfanilamide depressed the rate of sperm metabolism, increased sperm liveability, and partially inhibited bacterial growth. Fertility tests (62) showed that 0.3 percent (w/v) sulfanilamide in a yolk-citrate extender increased the nonreturn rate about five percentage units, a value that was soon confirmed (60). Not all reports were in agreement, particularly with antibiotics in the extender (57). Subsequent studies indicated that if the extender was mixed with semen before rather than after cooling to 5°C, the beneficial effect of sulfanilamide disappeared. This beneficial effect had been interpreted earlier (62) to be primarily a metabolic one, and the fact that egg yolk during cooling also was effective supported the idea that sulfanilamide enhancement of fertility was not due to bacterial inhibition. Sulfanilamide damages spermatozoa during the freezing process (163), and should be excluded from extenders used for semen that is to be frozen. Other sulfa drugs have been tested for possible superiority over sulfanilamide (162), but none has been found that warrants adoption.

Penicillin and Streptomycin. These two antibiotics were among the first to be researched intensively and are still in use. They were found to be relatively harmless to sperm cells and, particularly in combination, inhibited a broad spectrum of microorganisms (57, 123). Almquist (164) first reported that 500 to 1,000 units of penicillin per milliliter of extended semen increased the fertility of low-fertility bulls. Foote and Bratton (165) obtained higher nonreturn rates when 1,000 units of penicillin per milliliter, or 1,000 micrograms of streptomycin per milliliter, or both, were added to extended semen. Sulfanilamide had no effect. These findings were substantiated by other reports (166, 167). Later most of the beneficial effect of the two antibiotics was reported to be due to streptomycin in either egg yolk or milk extender (168, 169). All American reports showed that the nonreturn rate of low-fertility bulls tended to be raised more by addition of these antibiotics to semen extenders than did that of high-fertility bulls, and that delayed returns were decreased.

An extensive trial by Campbell and Edwards (67) also showed that fertility increased when sulfanilamide, penicillin, and streptomycin were added to yolk-citrate singly or in combination. Streptomycin depressed fertility in yolk-phosphate extender, probably because of a precipitate that was formed as a result of the reaction of the streptomycin calcium chloride complex with phosphate buffer. Easterbrooks et al. (170) observed that this problem can be circumvented by using dihydrostreptomycin. Extensive fertility testing indicates that the different forms of streptomycin give equal fertility.

Penicillin usually is added at levels of 500 to 1,000 units per milliliter and streptomycin or dihydrostreptomycin at 500 to 1,000 micrograms per milliliter of extender. Levels much greater than these have been found by some to reduce sperm survival (57), but levels as high as 4,000 units of penicillin and 8,000 micrograms per milliliter of streptomycin or the dihydro form were reported to be harmless (171). Conception rates were improved with 500 to 5,000 micrograms per milliliter of dihydrostreptomycin added to a yolk-citrate-sulfanilamide extender (172).

Both penicillin and streptomycin have been widely used since about 1950. Their popularity stems from the overwhelming evidence of their beneficial effects on fertility. Even the low percentage of pregnant animals that show estrus and are again inseminated are usually protected from possible abortion. Antibiotics have not shown beneficial effects in all tests, however. The semen in these tests may have been free from pathogens, or the method used to expose the semen to antibiotics may have been ineffective. The fertility response to antibiotic treatment today would probably be less than that originally observed now that many of the organisms, such as *Vibrio fetus* (which streptomycin particularly helped to control), have been eliminated from bull studs. In the Netherlands rigid culling of bulls based on bacteriological examination has long been the rule, and antibiotic treatment of semen has had negligible effects on fertility. On the other hand, Melrose et al. (114) in England reported a 3.4 percent increase ($P < 0.001$) in fertility when semen from *V. fetus*-free bulls was treated with 1,000 micrograms of streptomycin per milliliter.

Other Antibiotics. Many other antibiotics have been screened for effects on spermatozoa (see Table 16-11). Oxytetracycline (20 $\mu\text{g}/\text{ml}$), chlortetracycline (50 $\mu\text{g}/\text{ml}$), and chloramphenicol (500 $\mu\text{g}/\text{ml}$) in individual tests were ineffective in raising fertility (178). Several antibiotics such as the tetracycline series and particularly the fungicidal agents nystatin and amphotericin B are quite spermicidal. Others, such as epicillin, are relatively innocuous to sperm cells but also are ineffective against bacterial contamination. Nevertheless, an array of antibiotics exist that are effective against a variety of organisms. Since they can ordinarily be combined safely at levels that are harmless to spermatozoa when they are used singly, judiciously chosen combinations could inhibit a broad spectrum of semen contaminants. Lincomycin and spectinomycin have

Table 16-11. Nons

Antibiotic	M _{lev}
Tetracycline (Panamycin)	
Oxytetracycline (Terramycin)	<
Chlortetracycline (Aureomycin)	<
Chloramphenicol (Chloromycetin)	
Neomycin	
Polymyxin B	
Erythromycin	
Kanamycin	
Tylosin	
Thiostrepton	
Nystatin	
Amphotericin B	
Colymycin	
Lincomycin	
Spectinomycin	
Epicillin	
Ampicillin	

*Tests that were conducted at levels where toxicity may be greatly reduced.

^bYC = yolk-citrate; SN =

^cFoot et al. Unpublished.

Table 16-11. Nonspermicidal levels of antibiotics in bull semen extenders.^a

Antibiotic	Maximum safe level	Kind of extender and temperature ^b	Reference
Tetracycline (Panamycin)	75 µg/ml	Krebs-Ringer, 3 hrs. at 37°C	(36)
Oxytetracycline (Terramycin)	200 µg/ml	YC, 8 days at 5°C	(173)
	<125 µg/ml	SM, 4 days at 5°C	(174)
Chlortetracycline (Aureomycin)	100 µg/ml	YC, 4 days at 5°C	(154)
	62.5 µg/ml	YC, 15 days at 5°C	(171)
	<50 µg/ml	YC, 14 days at 4.5°C	(175)
	<250 µg/ml	SM, 4 days at 5°C	(174)
Chloramphenicol (Chloromycetin)	500 µg/ml	YC, 15 days at 5°C	(171)
	1000 µg/ml	YC, SM, 12 days at 5°C	(c)
	1000 µg/ml	YCG, YTG, months at -196°C	(c)
Neomycin	500 µg/ml	SM, 4 days at 5°C	(174)
	1000 µg/ml	SM, 20 days at 5°C	(176)
Polymyxin B	2000 µg/ml	YC, 14 days at 5°C	(154)
	1000 µg/ml	SM, 4 days at 5°C	(174)
	1000 µg/ml	YCG, YTG, months at -196°C	(c)
	1000 µg/ml	YC, 30 min. at 30°C	(177)
Erythromycin	1000 µg/ml	YC, SM, 12 days at 5°C	(c)
	1000 µg/ml	YCG, YTG, months at -196°C	(c)
Kanamycin	1000 µg/ml	YC, SM, 12 days at 5°C	(c)
	1000 µg/ml	YCG, YTG, months at -196°C	(c)
Tylosin	1000 µg/ml	YC, SM, 12 days at 5°C	(c)
	250 µg/ml	YCG, YTG, months at -196°C	(c)
Thiostrepton	25 µg/ml	YC, SM, 12 days at 5°C	(c)
Nystatin	5 µg/ml	YC, SM, 12 days at 5°C	(c)
Amphotericin B	<1 µg/ml	YC, SM, 12 days at 5°C	(c)
Colymycin	<250 µg/ml	YCG, YTG, months at -196°C	(c)
Lincomycin	1000 µg/ml	YCG, YTG, months at -196°C	(c)
Spectinomycin	1000 µg/ml	YCG, YTG, months at -196°C	(c)
Epacillin	1200 µg/ml	SM, 20 days at 5°C	(176)
Ampicillin	1000 µg/ml	YCG, YTG, months at -196°C	(c)

^aTests that were conducted without egg yolk or milk in the medium generally are excluded because toxicity may be greatly different in the absence of these ingredients.

^bYC = yolk-citrate; SM = skim milk; YCG = yolk-citrate-glycerol; YTG = yolk-tris-glycerol.

^cFootnote et al. Unpublished data. Cornell University. 1963-1973.

been combined to provide such control. This combination, along with clindamycin, is especially effective against mycoplasma (179).

Few fertility tests have been reported in recent years. The addition of polymyxin B has been reported to increase the fertility of frozen semen (180, 181); this is similar to the original report for liquid semen (165). Subsequent studies with both liquid and frozen semen have failed to demonstrate an improvement when added to extender containing penicillin and dihydrostreptomycin (see Table 16-11).

Other Chemotherapeutic Agents. Pyridium, phenoxethol (161), nitrofurans (36, 161), sodium azide (a respiratory poison) (182), and other agents (183) have been added to semen. These have either been relatively toxic to sperm cells at levels that are bacteriostatic, or they have been difficult to use routinely because of low solubility or instability.

16-5.3 Control of Specific Pathogens. Much attention has been given to the control of specific pathogens in semen (57, 148, 149, 182). Although unrecognized at the time, one of the organisms that could be spread widely by semen from infected bulls during the early expansion of artificial insemination was *Vibrio fetus*. Numerous studies (184-187) support the conclusion that dilution and streptomycin treatment of semen prevents vibriosis from being transmitted via AI by *V. fetus* carrier bulls. But Morgan et al. (119) showed that streptomycin treatment of semen extended with egg yolk was ineffective in controlling the spread of infection by AI, whereas in milk it was effective. Albertsen (182) showed that the marked effectiveness of streptomycin against *V. fetus*, *B. abortus*, and *Corynebacterium pyogenes* was greatly diminished by egg yolk. He recommended incubating the semen with antibiotic for 15 minutes at 37°C or for 60 minutes at 30°C before adding egg yolk. A modification of this incubation procedure (177, 180) with the addition of 1,000 units of polymyxin B plus 2000 micrograms of dihydrostreptomycin per milliliter of raw semen is reported to provide a more effective control of *V. fetus*. It is possible that the antibiotic neomycin would be a better choice to combine with penicillin and streptomycin because several strains of *V. fetus* are sensitive to this antibiotic (36, 174), whereas polymyxin B was less effective (174).

Similar examples could be given for other pathogens but the problems have been discussed thoroughly (148, 149). The fact is that many organisms can survive in frozen semen. Clearly the kind and quality of antibiotic, the time and temperature of exposure of organisms to the antibiotics, and the composition of the extender should be considered in formulating a satisfactory procedure for controlling microorganisms in semen.

The ultimate goal is to eliminate all harmful organisms from the bull stud and, through sanitary procedures, to minimize semen contamination. Even under the best conditions, however, an effective mixture of microbial inhibitors in the semen appears to be good insurance.

16-6 ACTIVATION

Early work on activation of spermatozoa has not been necessary to obtain a 68 percent activation rate. An analog of cell and spermatozoa at 4°C in 14 days (190). As a result of "Implementation of the hope of support the

The and improved spermatozoa when chilled pressed semen studies inhibit sperm dis proved s

Prot tetracycline amino acid Caproge (7,419 in extender increased with 8 Zealand units as insemination

16-6 ACTIVATORS, INHIBITORS, AND OTHER ADDITIVES

Early work on many types of additives has been reviewed (57), showing that activation by adjustment of pH has not given consistent results and should not be necessary in a properly buffered extender. The addition of sodium bicarbonate to Caprogen in a breeding experiment with 9,741 cows (87) resulted in 68 percent 49-day NR compared with 67.1 percent for the controls ($P > 0.05$). An analog of thiamine—thiamine propyl disulfide—is reported to penetrate the cell and 10 to 60 ppm added to yolk-citrate at 4°C markedly increased spermatozoa viability (188). Adenosine increased the motility of sperm stored at 4°C in 14G (Table 16-3) extender (189). At 37°C 6 millimoles caffeine stimulated and maintained motility of bull sperm cells without loss for four hours (190). Ascorbic acid is also reported to improve fertility periodically (191). "Implementors," such as posterior pituitary extract (mostly oxytocin) and a parasympathetic nerve stimulant (carbamylocholone), have been tested with the hope of aiding sperm transport. Limited experiments in Russia (91) tend to support their value but the results are often not repeatable (192).

The tranquilizer chlorpromazine, at 200 µg/ml, could replace sulfanilamide and improve sperm motility in egg yolk extender (CU-16), but it is toxic to spermatozoa in skim milk. In extensive field trials fertility was not affected when chlorpromazine was added to egg yolk extender (75). Promazine depressed sperm motility at levels as low as 20 µm/ml. Other tranquilizers have been studied (193) and incorporated into the Japanese commercial liquid semen extender, Neoseminan (95). Carbon dioxide and reduced pH also may inhibit and preserve spermatozoa (see Sections 16-2 and 16-4). At very high sperm dilutions cyanide, a highly toxic chemical that inhibits oxidation, improved sperm survival at 35°C in a fructose-containing medium (43).

Protective agents added include the chelators EDTA (ethylenediamine tetracetic acid) and glycine (89, 194, 195). EDTA also protects against the amino acid oxidase released by dead sperm (196), but when it is added to Caprogen (87) fertility is significantly lower (4.8 percent) than with catalase (7,419 inseminations). Catalase is included routinely in ambient temperature extenders to hasten the decomposition of H_2O_2 . In one report catalase alone increased sperm survival but failed to increase fertility except when combined with 8 µg/ml of α -amylase (197). According to several reports from New Zealand, however, catalase increased the nonreturn rate 1 to 2 percentage units as indicated by the following 49-day NR data based upon 669,639 inseminations:

Age of semen	Caprogen	Caprogen + catalase
Day collected	63.4%	64.6%
Day later	66.0%	68.1%

In another trial (196) catalase increased the nonreturn rate 3.1 percent on 8,154 services. Total sperm numbers per insemination dose in Caprogen plus catalase with 5 percent egg yolk could be reduced, without reducing fertility, from 2.5 to 1.5×10^6 .

Catalase improves sperm survival at 5°C as well. The effect is most pronounced at the higher extensions of semen (45, 198, 199). Catalase is superior to peroxidase in protecting bull spermatozoa (199). With semen used for insemination at 5°C catalase (1 to $20 \mu\text{g}/\text{ml}$) added to CUE failed to improve fertility (40, 85) although there was a suggestion of a beneficial effect on fertility after 4 days' storage (45). Catalase improved fertility in heated homogenized whole milk as indicated by the following data (80).

	No catalase		1 μg catalase/ml	
	CUE	Milk	CUE	Milk
No. inseminations	6,064	5,944	6,243	5,586
% 60-90-day NR	72.9	68.0	73.2	71.6

Hyaluronidase was thought to play a role in the sperm's ability to penetrate the egg, but hyaluronidase added to semen failed to give a consistent response (57). More recently α - and β -amylase and β -glucuronidase have been shown to improve sperm motility (200, 201). Fertility tests with frozen semen are described in Chapter 17. In New Zealand tests (13,082 inseminations) with semen handled at ambient temperatures α -amylase alone added to Caprogen gave 63.4 percent versus 64.6 percent 49-day NR for control semen (183). The addition of nicotinamide, which as a competitor of nucleosidase in plasma reduces nicotinamide adenine dinucleotide (NAD), significantly depressed fertility 1.9 percent (87).

Hormones have been added to semen in an attempt to influence possible sperm transport in the female, sperm capacitation in the cow, and ovulation time. When 200 IU of human chorionic gonadotrophin (HCG) were added to semen to influence ovulation time fertility results were poorer (202).

	No. cows	% 60-90-day NR
Milk	2,077	64.6
Milk + HCG	1,999	62.8

The administration of female sex hormones estrogen and progesterone to extended semen has also been ineffective in altering fertility (57, 203). In conclusion we can say that catalase is the only additive that has been tested thoroughly and used frequently, particularly when semen is stored at ambient temperatures.

16-7 EXTEN

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16-7 EXTENSION PROCEDURES AND RATES

Semen should be processed in a manner that will insure maximum fertility. The final extension rate of semen should be planned so that the number of sperm present in a package convenient for handling and insemination will be sufficient but will not waste sperm cells unnecessarily.

16-7.1 Initial Semen Extension. It is important to mix bull semen with a preserving medium relatively soon after semen collection in order to provide spermatozoa with necessary ingredients and to protect them in various ways (Section 16-2). Semen and extender should be combined at the same temperature, usually about 30°C. The possible importance of a short incubation period at this temperature with antibiotics added to insure adequate bacterial control is discussed in Chapter 17. For semen used at ambient temperature the final extension can be made at one time.

When semen is to be cooled to approximately 5°C it is convenient to cool the extended semen in relatively small volumes and then add more cold extender to achieve the final extension rate. This can be accomplished by mixing the neat semen with 2 to 4 times its volume of extender before cooling to 5°C and then adding cold extender. Semen cooled without the benefit of extender was 6.5 percent lower in fertility (55) than semen mixed before cooling, clearly indicating the need for protection against the cold. When sperm cells become cold shocked they exhibit a decrease in motility, reduction in metabolism, loss of key components, and morphological damage.

16-7.2 Cooling Rates. Cooling rate undoubtedly is important (see Chapter 17), but the optimum rate for most extenders has not been tested with regard to fertility. Rapid cooling is generally undesirable. McFee and Swanson (204) found that sperm motility was considerably improved during a 10-day storage period at 5°C when sperm in a yolk-citrate-fructose extender were cooled from 32 to 5°C in 140 minutes compared to 25 minutes. An extensive fertility test with homogenized, sterilized 9 percent cream (205) gave the following results:

<i>Cooling time from 30° to 4°C</i>	<i>No. cows</i>	<i>% 60-90-day NR</i>
120 minutes	9,163	64.8
40 minutes	8,659	63.0

The slower cooling was definitely preferable ($P < 0.02$).

16-7.3 Storage Temperature. Unfrozen semen (except for that which is stored at ambient temperature) is almost always stored at 4 to 5°C. This temperature can be maintained fairly easily with ice and has been satisfactory for

semen used to impregnate millions of cows. It may not be the optimum holding temperature, however. Swanson et al. (206) found significantly better motility and fertility in a yolk-citrate-bicarbonate-fructose extender used to store semen at 10°C rather than at 4 to 5°C. When 1,338 cows were inseminated with semen stored for 3 to 4 days at 4 to 5°C and at 10°C the 60-90-day NR figures were 67.9 and 73.5 percent respectively. Storage at 10°C has not been used commercially, however, because it is difficult to maintain this temperature under practical conditions.

16-7.4 Final Extension Rate and Sperm Numbers. Semen for use at 5°C is usually extended to give the desired number of sperm cells for insemination in one milliliter. Although there is little difference in fertility when 0.5 to 2.0 milliliters of extended semen is inseminated (139) the volume used most often is 1.0 milliliter. Figure 16-1 shows a summary of work by Salisbury and co-workers and Willett and others prior to 1960. In the pioneer work by Salisbury (207) fertility sometimes appeared to increase as semen was extended at higher

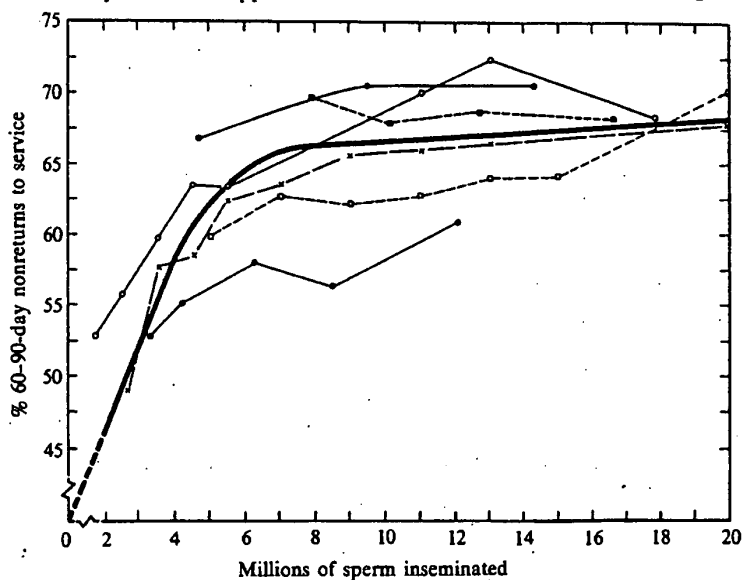


Figure 16-1. A summary of several of the investigations made to determine the minimum number of spermatozoa for optimum fertility in cattle. Most of the experiments were conducted with yolk-citrate as the diluent, with and without sulfanilamide and antibiotics. [From Salisbury and VanDemark. *Physiology of Reproduction and Artificial Insemination of Cattle*. 1st ed. W. H. Freeman and Company, San Francisco. 1961.]

rates up to 1. As Figure 16-1 shows, sperm insemination with spermatozoa at a range of sperm counts and Larson's rate for each sperm count diminished from 66.7 percent for a sperm count of more than 10 million to a maximum concentration of 66.7 million motile sperm per milliliter were recom-

Since the optimum fertility is possible by several trials indicate that when changed little extremely live sperm sufficient to prove the does it correct. (The latter

In an experiment per inseminationally and in extended numbers of percentage up from 2.5 to 5 percent to a reduction per land is 0. required reduced

The liquid ser-

rates up to 1:100. Dilution may have helped to control bacterial contaminants. As Figure 16-1 shows, there is a curvilinear relationship between number of sperm inseminated and fertility. The data are based upon extending the spermatozoa at different rates regardless of initial sperm concentration. This gives a range of sperm concentrations per milliliter used for insemination. Willett and Larson (167) calculated that there was a 0.6 percent decline in nonreturn rate for each decrease of one million sperm inseminated as the numbers diminished from 12 to 4 million. Below 4 million the nonreturn rate declined 3.9 percent for each reduction of one million spermatozoa. In general, an extension rate of more than 100 $\times$ was possible before too few sperm were present for maximum conception rates. Bratton et al. (208) took initial sperm cell concentration and motility into account and extended samples to 5, 10, and 15 million motile cells per milliliter. This resulted in 60-90-day NR figures of 66.7, 70.9, and 70.5 percent respectively. Therefore 10×10^6 motile sperm were recommended for insemination.

Since 1960 the number of sperm per insemination necessary to maintain optimum fertility with liquid semen has decreased further. This has been made possible by the development of more balanced extending media. Results of several trials in this development are shown in Table 16-12. The trials at 5°C indicate that 4 million motile sperm per milliliter gave initial high fertility and that when the number per insemination was decreased to 2×10^6 fertility changed little (109). A report by the British Milk Marketing Board showed that extremely high dilutions of semen from two bulls up to 1:3,000—giving 500,000 live sperm per milliliter—resulted in 32 and 46 percent conception. When sufficient sperm are inseminated increasing the number further does not improve the fertility of poor quality semen (210) or low-fertility bulls (15), nor does it compensate for the reduced fertility caused by poor technicians (87). (The latter effect was studied at ambient temperature.)

In ambient temperature studies (see Table 16-11) 12.5 to 25×10^6 sperm per insemination originally were employed. This number was reduced drastically and fertility remained satisfactory. Reducing the proportion of egg yolk in extender from 20 percent to 5 percent facilitates extending to very low numbers of sperm. Shannon (138) observed that fertility declined 10.9 percentage units when the sperm dose in 20 percent yolk-Caprogen was reduced from 2.5 to 0.5×10^6 sperm per insemination. The corresponding decline with 5 percent egg yolk was only 3.1 percentage units. This decline is probably due to a reduction in sperm numbers below the critical level, although high dilutions per se could be detrimental. Since the volume inseminated in New Zealand is 0.5 milliliter the actual semen dilution is one-half of what would be required if one milliliter were used. Nitrogen in the Caprogen extender also reduced the dilution effect (46).

The enormous impact that a single sire could have on the gene pool in a liquid semen program, if enough cows were available to inseminate with

Table 16-12. Fertility of unfrozen semen with different numbers of spermatozoa per insemination.

Temperature and treatment	No. 1st services	% 60-90-day NR	Reference
5°C, motile sperm			
CUE, 5×10^6 sperm	24,737	75.2	(5)
CUE, 10×10^6 sperm	18,933	76.1	
CUE, 4×10^6 sperm	11,065	72.7	(15)
CUE, 8×10^6 sperm	9,739	73.4	
Tris, 4×10^6 sperm	11,445	72.7	
Tris, 8×10^6 sperm	10,071	74.1	
Ambient; total sperm ^a			
Caprogen, 5×10^6	15,997	68.0	(196)
Caprogen, 3.75×10^6	8,574	69.3	
Caprogen, 2.5×10^6	8,922	67.0	
Caprogen, 3.75	14,110	63.6	(196)
Caprogen, 2.5	6,130	63.3	
Caprogen, 1.875	6,124	61.2 ^b	
Caprogen, 2.5	12,676	66.8	(87)
Caprogen, 0.5	4,114	59.5 ^c	

^aSperm concentration/ml is twice that given; 0.5 ml is used for insemination.

^b $P < 0.05$.

^c $P < 0.01$ within experiments.

0.5×10^6 total sperm, is obvious. About 30 billion or more sperm per week can be collected from most mature healthy Holstein sires. This number of spermatozoa is sufficient to inseminate 6,000 cows per week. In New Zealand 2.0 to 2.5×10^6 total sperm are routinely used per insemination, except when frozen semen has been banked for later thawing and used as needed at ambient temperatures. In this case, the semen is thawed and reconstituted in Caprogen with 20 million sperm per insemination dose.

16-7.5 Packaging Unfrozen Semen. All semen should be packaged in opaque containers for transport and storage in order to prevent exposure to light (33). In moderate climates where ambient temperatures are used the semen is often wrapped in aluminum foil and placed in light-weight styrofoam containers. The containers should be closed and kept out of direct sunlight.

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NR	Reference
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For semen stored at 3 to 5°C a plastic bottle of frozen water included in a styrofoam or other insulated container will keep the cells at this temperature as long as there is ice in the water. The bottle of frozen water should be removed from the freezer, where it has been held several degrees below freezing, to allow it to warm up from freezer temperatures to 0°C before placing the ice and semen together in the container. It is safe to combine them as soon as the ice in the bottle starts to melt. If the ice bottle is not allowed to defrost it could be cold enough to freeze and damage the spermatozoa.

A similar danger is encountered in winter when the inseminating tubes may be chilled by exposure to below-freezing temperatures. Cold shock results when the diluted semen held at 5°C is moved from its container into inseminating equipment that has been chilled by exposure to freezing weather conditions.

If the temperature of the diluted semen rises during shipment, more ice or more insulation or both are needed. It is not always possible to add more ice to the package, in which case containers with better insulation are required, especially in hot weather.

16-7.6 Warming Rate. After extended semen has been stored at approximately 5°C the rate of warming the cells to body temperature is not critical. The temperature can be raised very rapidly from 5°C to body temperature without harming the cells.

16-8 PROCESSING SEMEN FOR SEX CONTROL

Innumerable reports have appeared concerning alteration of the sex ratio (211, 212). Limited control appears to be possible, but no experimental data have been found to support claims by commercial concerns that they are able essentially to guarantee the sex of the calf produced.

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17

Principles and Techniques of Freezing Spermatozoa

17-1 INTRODUCTION

All life proceeds in a continuous stream, the individuals in it constituting a part of the linkage that has joined past and present life forms and will ultimately join present life forms with those of the future. The life span of each link in the chain is limited by its genetic makeup and its environment, some part of which is controlled by the metabolic rates of the individual links. Among the processes that control the metabolic rate at which spermatozoa exhaust themselves is the temperature at which their life-sustaining chemical processes proceed. These chemical processes generally take place within a measurable temperature range and vary within that range in direct response to the temperature.

Water is the primary constituent of biological fluids responsible for the internal transport of essential chemicals. Pure water freezes and forms crystals at 0°C, whereas water containing ions and other substances in solution freezes at lower temperatures depending on the concentration of these substances. As water in a solution is frozen, pure water crystals form leaving behind greater liquid concentrations of those substances in solution. This increases the osmotic pressure of the remaining solute, which can damage the cells.

British workers discovered that glycerol protected spermatozoa against some of these hazards when it was added to the extender solution in which the spermatozoa were suspended. Since then, freezing has been the preferred method of preserving spermatozoa.

Spermatozoa can now be stored in liquid nitrogen at temperatures as low as -196°C and survive with relatively high fertility after thawing. However, many of the contained spermatozoa are killed or rendered immotile by freezing and thawing. Hence, for optimum fertility larger numbers of spermatozoa are used for frozen than for unfrozen semen.

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Egg yolk and/or milk are the basic ingredients of most extenders for semen freezing. Other protective compounds similar to glycerol have been tested and found unsatisfactory in one way or another. The fertility of frozen semen depends greatly on the care exercised in the initial extension of the semen before freezing, the method of adding the glycerol, the time permitted for equilibration of the spermatozoa with the glycerol, the rate of freezing, and the stability of the temperature during storage. After storage fertility depends on the care and method of warming the extended semen before insemination. When proper procedures are used fertility rates equal to those of any other process can be maintained for extended periods.

Although many of the procedures for freezing spermatozoa are relatively simple, they must be performed correctly if the highest potential fertility for insemination is to be maintained. This chapter will acquaint the reader with the present state of the art of preserving bovine semen by freezing and will relate that to the latest research dealing with the process.

17-2 THEORETICAL ASPECTS OF FREEZING AND THAWING

17-2.1 Physical and Chemical Consequences. At least 50 percent of the sperm from an unselected population of bulls are killed or rendered immotile during freezing and thawing. The damage is caused 1) by internal ice crystal formation that affects the structure of the spermatozoa, 2) by the solute concentration that results as pure water is withdrawn from suspension media both inside and outside the cells, and 3) by the interactions of these two physical factors. This theory of cellular damage is widely accepted among cryobiologists as a working hypothesis in research in this field.

The major physical and chemical consequences of freezing are the removal of pure water from solution to form ice and the resultant increased concentration of solutes in the residual liquid. These events and their effects on the cells are influenced by the level and types of cryoprotective agents, the osmolality and pH of the extender, and the freezing rate (1, 2, 3, 4). Spermatozoa to be stored in glass ampules have generally been frozen at relatively slow rates. The range of freezing rates employed has been about 0.8 to 3°C per minute from +5 to -15°C, and 3 to 5°C per minute from -15 to -79°C. These rates result in the formation of a relatively small number of large extracellular ice crystals.

The freezing point of a solution is based on the concentration of particles it contains as solutes. A given molecular weight of an electrolyte that dissociates into ions on entering into solution will lower the freezing point more than the same proportionate weight of a molecule that does not dissociate. As a solution cools to, through, and past the point at which ice forms, a continuing series of events takes place. The rate of cooling depends on the difference in temperature between the solution and its coolant and their relative

mass. Depending on the concentration of solutes and the rate of cooling, the temperature of the solution falls below the true freezing point of water until an ice crystal forms around a "seed." At that point the heat of fusion required to form ice must be removed from the solution, so its temperature shifts upward as the process takes place. As each molecule of pure water freezes out of the solution, the concentration of particles in that which remains increases, resulting in a continuous drop in the freezing point of that solution. These inter-related changes continue until the entire solvent and its contained solutes and other contents have completed the process through the eutectic point. Due to the longer exposure of the cells, the damaging effects of high solute concentrations should be most critical with slow rates of freezing. Similarly, because cells may be exposed to damaging solute concentrations during rewarming, fast thawing should be beneficial.

Although the formation of extracellular ice crystals was once considered an important disruptive force it does not seem to be a primary cause of sperm-cell damage during freezing (6, 7). Extracellular ice is not, in fact, particularly harmful (6-9). With few exceptions (10, 11), however, intracellular ice is lethal (1, 3, 5, 6). In addition, recrystallization—the growth of large crystals at the expense of smaller ones—can occur at temperatures above -130°C (2). Ice crystals can also form and cause cell damage as stored samples are warmed from -196°C .

The increased solute concentrations created as water freezes appear to be a major cause of cell damage. For example, hemolysis of erythrocytes occurs at electrolyte concentrations above 0.8 M NaCl regardless of temperature (12), and high salt concentrations damage bull spermatozoa (13). During freezing the temperature at which bull sperm were damaged was found to be lower in a hypotonic extender than in an isotonic one (14). Solute concentration, the rapidity of its change, and the damaging effects on spermatozoa depend on temperature (3, 5, 8) and its rate of change. During slow freezing, the cells are exposed for prolonged periods to concentrations of solutes that are increasing relatively slowly and some cellular dehydration may occur. With rapid freezing the length of exposure to increased solute concentrations is reduced and intracellular dehydration may not occur.

The increase in osmotic pressure of the suspension medium that accompanies freezing pulls water from within the cell, causing intracellular dehydration with an attendant increase in intracellular electrolyte concentration. In contrast, rapid freezing, such as that obtained by direct immersion in liquid nitrogen with cooling rates that probably exceed 200°C per minute, results in the formation of a relatively large number of small crystals both within the spermatozoa and in the suspension fluid. Intracellular ice can form during rapid freezing because there is insufficient time for all freezable water to diffuse from the cell (4, 5). Both rapid and slow freezing cause cellular damage, although different mechanisms appear to be responsible.

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Changes in pH have been observed during freezing caused by precipitation of salts in saturated solutions (15, 16). This could influence cellular survival, although under normal conditions it seems unlikely that solutions sufficiently saturated to change pH so as to harm the spermatozoa would be produced until after their own metabolism had been greatly slowed by the drop in temperature.

The exact nature of total freeze-thaw damage is not fully understood. It has been suggested that freezing damage is a consequence of the vapor pressure differential between ice and water at temperatures below 0°C. The theory is that the greater vapor pressure of the water would cause its redistribution, leading to injury from dehydration during slow freezing, and to membrane rupture during rapid freezing (22). There is considerable evidence that cellular membranes are involved. Saacke and Almquist (17) observed loss or damage to the cellular membranes of spermatozoa frozen without glycerol, and Rapatz (18) observed damage to the sperm neck, due to intracellular ice, following rapid freezing. Pickett and Komarek (19) found that lipid was lost from spermatozoa during freezing, presumably leaching from cellular membranes. Membrane damage may also account for the increased leakage of sperm enzymes following freezing without glycerol (20). Decreased mitochondrial enzyme activity has been noted following the freezing of mouse liver (21). Such changes are presumably the results of increased solute concentrations that induce protein denaturation.

17-2.2 Glycerol Function. The cryoprotection afforded when glycerol is added to seminal extenders was originally attributed to changes in the size and shape of ice crystals formed (6, 9, 18)—changes that presumably reduced their mechanical destructiveness. A more popular hypothesis states that glycerol acts through a "salt-buffering" mechanism (12). Since glycerol binds water (3, 23, 24) and markedly decreases the freezing point of solutions, less ice forms in its presence at any given temperature. Consequently, the solute concentration in the residual liquid is correspondingly reduced. The damaging influences of concentrated solutes appear to depend on temperature (3, 6, 8); hence glycerol, by reducing the temperature at which such solute concentrations are attained, presumably reduces their damaging effects. In support of this concept, the optimum glycerol level of an extender increases as extender tonicity is increased (25, 26, 27). Glycerol probably dehydrates the cells (34, 35) and it forms complexes with metallic ions (33).

Although cellular damage can be caused by concentrated solutes, other unknown sources of damage presumably exist, and knowledge of the site of glycerol action is essential to a critical evaluation of both cryoprotective mechanisms and the nature of freezing damage.

It has generally been believed that glycerol enters live spermatozoa suspended in it and does so rapidly. Glycerol is oxidized by sperm cells (28, 81) and it appears in autoradiographs to concentrate in the posterior part of the

head (29). The rate of entry is not known, however; nor is it known whether glycerol buildup within the cell is necessary for protection against freezing damage (31, 35). The early view in support of an intracellular glycerol requirement was based on work with red blood cells (12), but recently has been shown not to hold in tests of bovine sperm that compare their freezability after momentary and 30-minute exposure to glycerol before freezing (35). Spermatozoal survival was greatest in one study (34) when they were frozen after only momentary exposure to glycerol rather than after 30 minutes' to 6 hours' exposure. Bull sperm cells have been successfully frozen in the presence of cryopreservatives that do not enter the cell if the freezing rate is very rapid (32). To be most effective, glycerol requires relatively slow freezing rates.

Since spermatozoa act like osmometers, swelling in hypotonic extenders as water enters the cells and shrinking in hypertonic extenders as water is withdrawn from them, it was thought that this technique could be used to determine the rate at which glycerol entered into spermatozoa. The presence of 7 percent glycerol in an isosmotic extender containing a soybean protein decreased spermatozoan volume. In two concentrations of citrate dramatically different in osmolality, in the presence or absence of egg yolk, the packed cell volume was increased by 7 percent glycerol. The change recorded was as great 5 minutes after as it was 30 minutes after the addition of glycerol and was, of course, greater in the hyposmotic extender (30).

17-23 Other Cryoprotective Agents. Glycerol for the freezing of bull semen remains the standard of comparison for any cryoprotective agent. No substitute has yielded results as satisfactory. A number of related compounds, including ethylene glycol and propylene glycol have been investigated (36, 37, 38). Dimethyl sulfoxide (DMSO) penetrates cells rapidly, has a "salt-buffering" effect, and is most effective during slow freezing. A number of disaccharides and trisaccharides have also shown some cryopreservative effects. They do not penetrate the cell and seem most effective when used with relatively fast freezing rates (32). A number of theories have been advanced to explain the cryoprotective effects, most of which have to do with elimination of electrolyte damage (39), cell membrane permeability (40), possible preferential leakage of solutes (41), and buffering by nonelectrolytes (32).

17-3 EXTENDERS FOR FREEZING

17-3.1 Basic Components of Extenders for Freezing. The basic components of extenders for freezing bull spermatozoa are: 1) water (42) which is a solvent for seminal and extender components; 2) dissolved ionic and nonionic substances to maintain osmolality and to buffer the pH of the medium; 3) organic materials with the capacity to prevent cold shock (generally egg yolk or milk); 4) cryoprotective agents such as glycerol or DMSO; 5) simple sugars for an

energy source (82)
6) additives such as
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energy source (82) or di- and trisaccharides as added cryopreservatives; 6) additives such as enzymes that may improve fertility; and 7) antibiotics to control microbial growth (see Chapter 16).

Buffers and Nonionic Substances. A variety of buffers and nonionic substances have been used, among which are sodium citrate dihydrate (43, 44, 45), Tris (hydroxymethyl) amino methane (27, 46), monosodium glutamate (47), buffers with combinations of salt solutions (48) or carbohydrates (32, 35, 44, 49-52), and various milk preparations (25, 45, 46, 53, 54). Although a number of zwitter ionic buffers have received recent attention (55, 56), yolk-citrate remains the most widely used seminal extender. Reports of superior survival and fertility in other extenders are numerous (47, 51, 52, 54), but possible interactions with other factors in seminal processing make it impossible to generalize these findings; a satisfactory extender for one animal or one set of conditions may not be acceptable for others. Thus each laboratory must determine which extender, under its conditions, will provide maximum reproductive efficiency. It should be emphasized that almost all recommendations regarding minimum sperm numbers, the effects of long term storage, and so on, are based on results with yolk-citrate extenders. Each promising new extender or additive should be carefully evaluated before it is adopted for general use.

Organic Materials. Since Phillips (57) discovered that egg yolk was a valuable constituent in preserving fertility it has been used extensively in seminal extenders. VanDemark et al. (37) investigated the influence of egg yolk levels on the post-thaw motility of spermatozoa in a citrate extender; the results are shown in Figure 17-1. The highest survival was obtained with 24.2 percent egg yolk, although a second experiment showed that 16 percent egg yolk was slightly better than 24 percent. Levels of approximately 20 percent egg yolk have become standard in most extenders.

Post-thaw motility (58, 59); sugar utilization (59), lactic acid production (59), and fertility (58) were greatest when egg yolk was included in both fractions of the seminal extender—that containing no glycerol (which is added for cooling above freezing) and the remainder containing glycerol (which is added before the below-freezing cooling phase is begun).

Extenders have also been developed that consist primarily of heated skim milk and glycerol. With reconstituted skim milk the optimum combination of these components was 10 percent dry milk solids to 9.7 percent glycerol. With fresh skim milk, 10 to 13 percent glycerol was best (25, 54).

The beneficial effects of egg yolk and milk for preventing cold shock prompted research on the nature of the active constituents. It was found that egg yolk does not permanently alter the spermatozoa (60). The optimum levels of its protective constituents—lipoprotein, phospholipid, and lecithin (60-65)—in extenders range from 1 to 5 percent lipoprotein, 0.5 to 1.0 percent

phospholipid, and 0.25 to 2.0 percent lecithin (see Figure 17-2). The milk protein—casein—is the agent in milk responsible for preventing cold shock (62, 65, 66) and its optimum levels have ranged from 1 to 8 percent. The nature of cold shock prevention by these agents is not understood.

Cryoprotective Agents. Glycerol is the most widely used cryopreservative for bull spermatozoa. When frozen by conventional methods in glass ampules, approximately 7 percent glycerol is optimal for citrate-yolk and Tris-yolk extenders; 11 to 13 percent for fresh and reconstituted skim milk. The optimum glycerol level was lower with the pellet method of freezing (35, 51, 67). In fact, yolk-lactose and yolk-raffinose extenders without glycerol resulted in a 50.5 and 59.3 percent 60-to-90-day nonreturn (NR) on 32 and 59 first service inseminations, respectively. Partial cryoprotection (51, 67) was contributed by these sugars and they complemented glycerol when used in combination. When used individually glycerol was superior to dimethyl sulfoxide (DMSO) (68-70). Combinations of these cryopreservatives were not beneficial in homogenized and skim milk extenders. The addition of 1 percent DMSO to a yolk-Tris extender with 6 percent glycerol improved post-thaw motility. In a 7 percent glycerol-Tris-yolk extender, additions of 0.5 to 2.5 percent DMSO did not significantly improve liveability but favored the combined use of these cryopreservatives.

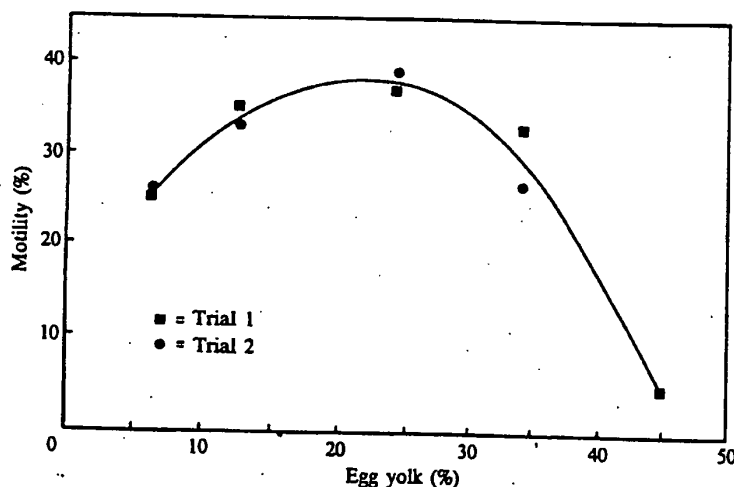


Figure 17-1. Percentage of motile spermatozoa after freezing and thawing of semen in diluents containing various levels of egg yolk. [VanDemark et al. Illinois Agr. Exp. Sta. Bull. 621. 1957.]

17-3.2 Sugar which sperm studied exter 54, 58, 64, 83 tion, which immediately troytes in m cent fructose whether the cooling or fr 85).

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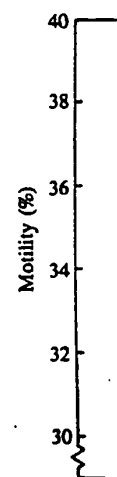


Figure 17-2. lecithin, ph. Sci. 53:81

17-3.2 Sugars. The monosaccharides—glucose and fructose—are sugars from which spermatozoa may derive energy for their life processes; they have been studied extensively as adjuvants to extenders for freezing spermatozoa (25, 46, 54, 58, 64, 83, 84, 85). Some improvement has been observed from their addition, which suggests that the improvement resulted not only from providing immediately available substrate (54) but from providing substitutes for electrolytes in maintaining the osmotic balance of the extender. A level of 4 percent fructose was excessive in one investigation (83). It does not seem to matter whether these sugars are added to either fraction of the extender used for cooling or freezing (48), but they may affect optimum equilibration time (46, 85).

The di- and trisaccharide sugars, however, which spermatozoa are not able to metabolize, provide their cryoprotective benefits primarily as relatively large molecules that contribute to osmotic balance by acting as substitutes for electrolytes. They are not capable of passing across the sperm-cell membranes during freezing or thawing and they probably diminish the effects of solute concentration. They give best results when added to semen in the glycerol-containing fraction before freezing and they are not contained in the extender

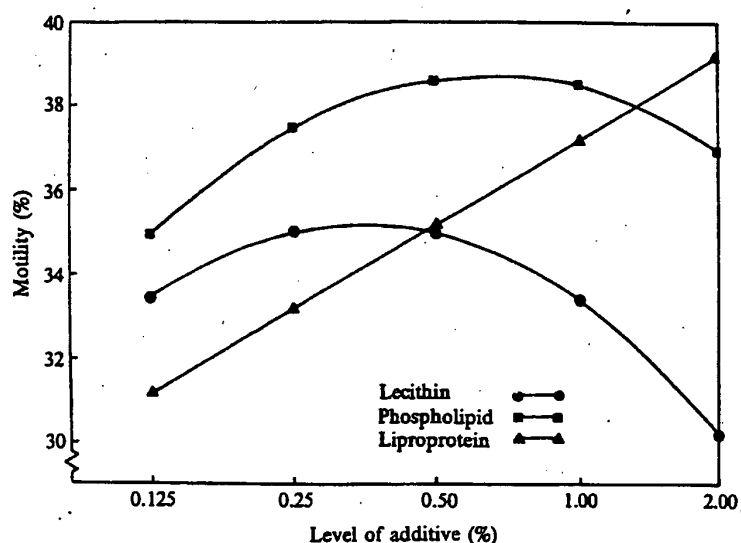


Figure 17-2. The post-thaw motility of spermatozoa extended in Tris-buffered lecithin, phospholipid, and lipoprotein extenders. [From Gebauer et al. *J. Dairy Sci.* 53:817-823. 1970.]

used for prefreeze cooling from body temperature (58). For pellet freezing, in which the rate of freezing is rapid, these sugars replace effectively the buffering salts of extenders (67) and provide cryoprotection for all (35) or part of the replaced glycerol (44) in tests based primarily on post-thaw motility. The fertility of semen frozen with sugar as the cryoprotective agent in yolk was lower than when some glycerol was included (32). The sometimes conflicting results reported in the literature involve differences in equilibration times, rates of freezing, and the kind of seminal containers used in freezing. The protection afforded by the large sugar molecules is different from that provided by glycerol and the two are not interchangeable. The data indicate that at this stage in our understanding glycerol is indispensable and sugars are sometimes complementary and useful.

17-3.3 Osmolality. Osmolality—the number of particles suspended as solutes in a solution—influences not only the osmotic pressure of a solution but the point at which the solvent freezes. Thus the lowered freezing point of a solution reflects its osmolal concentration of particles. An osmolal concentration of a solute in water depresses its freezing point 1.86°C . This is usually expressed as milliosmols; one milliosmol depresses the freezing point 0.00186°C . The freezing point depression of bull semen is approximately -0.55°C , equivalent to a concentration of about 300 milliosmols, which increases as the spermatozoa metabolize nutrients (71, 73). Thus evidence indicates that it is better to err on the side of hypotonic than hypertonic extenders (77). Results of a number of studies show that seminal extenders that are isosmotic with semen preserve the life of spermatozoa best when stored above or below freezing temperatures (14, 27, 71, 74, 75). Figure 17-3 illustrates the results of freezing semen in yolk extender made up of varying levels of sodium citrate. Clearly the effects of osmolality on spermatozoan liveability and ultimate fertility interact with a wide range of other variables, including the hydrogen ion concentration measured by pH (27, 76).

17-3.4 pH. The optimal pH for a seminal extender is influenced by its nature and by the ions it contains. The optimal pH for a phosphate buffer is 7.5 (76), and that for Tris and citrate extender is 6.5 to 7.0 (27) (see Table 17-1). The optimal pH appears to differ, however, at different stages of the semen processing procedure. Steinbach and Foote (27) noted that the least damage before freezing occurred in an extender at pH 6.5, but the smallest loss of spermatozoan motility during freezing was observed in an extender with a pH of 7.5. This was consistent with earlier findings of Pickett et al. (79). With unfrozen spermatozoa a decrease of 0.1 pH unit in a CUE extender increased the NR figures by 2.58 percent over the pH range shown in Figure 17-4. Freezing injury may be reduced in the presence of lower concentrations of hydrogen ions than are optimal for storage in the unfrozen state, and in Tris buffer high

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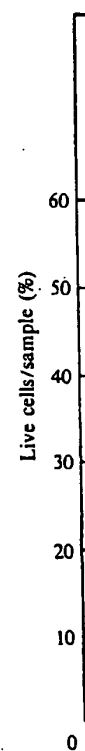


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pH was more toxic at low than at high osmolality of the buffer (27, 76). The pH may change during storage but the change bears little relationship to post-thaw motility (76, 80).

17-3.5 Enzymes. Spermatozoan capacitation (see Section 4-6.1), which occurs naturally in the uterus preparatory to fertilization, may occur at least partially *in vitro* by incubation with enzymes hydrolyzing long-chain carbohydrates (86). One of these enzymes is amylase, which is available commercially in either the alpha (α) or beta (β) forms. Most of these discoveries, and tests of the fertility of semen treated with amylase, have been made by Hafs

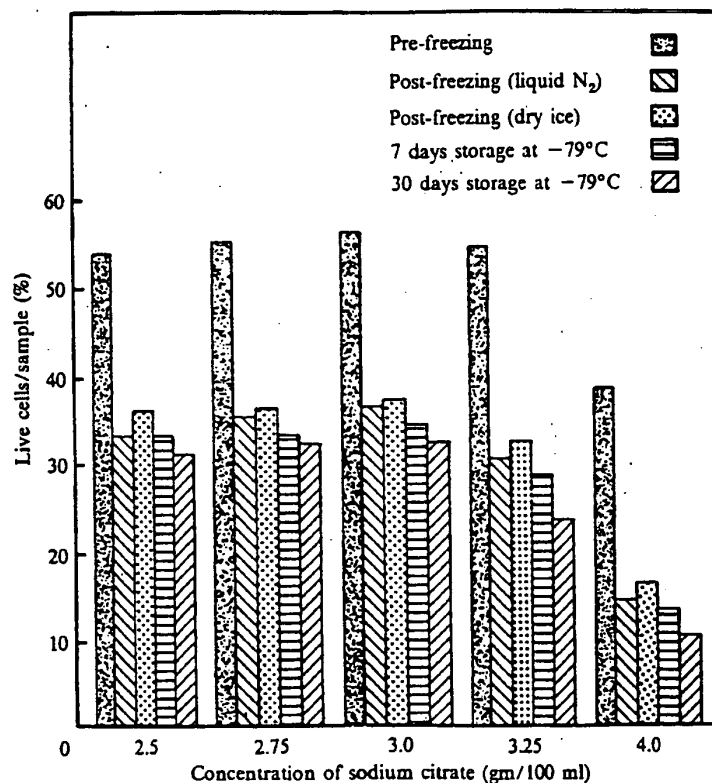


Figure 17-3. The influence of sodium citrate concentration upon the percentage of live spermatozoa at various intervals during processing and storage. [From Rao et al. *Fert. and Ster.* 19:129-136. 1968.]

Table 17-1. The influence of processing techniques and pH on fertility of bovine spermatozoa frozen in Tris and citrate extenders.^a

pH	Item	Tris ^b			Total or mean
		I	II	III	
6.5	1st services	1,363	1,491	1,083	3,947
	% NR	74	73	74	74
6.75	1st services	1,320	1,499	1,165	3,984
	% NR	73	72	72	72
		Citrate ^c			
		I	II	III	
6.5	1st services	2,615	2,454	2,464	7,533
	% NR	70	72	69	70
7.0	1st services	2,516	2,478	2,438	7,432
	% NR	70	70	69	70

^aFrom Foote. *J. Dairy Sci.* 53:1478-1482. 1970(78).

^b I. All samples glycerolized upon cooling and frozen 6 hours later.

II. Extenders contained glycerol initially.

III. Extenders contained glycerol initially, and samples were frozen 3 hours after cooling.

^c I. Four-time glycerolization immediately after cooling.

II. One-time glycerolization immediately after cooling.

III. One-time glycerolization 5 hours after cooling (1 hour before freezing).

and his associates at the Michigan Agricultural Experiment Station. Additions of from 1 to 20 micrograms of crude α - or β -amylase generally increased fertility (87, 88, 89), although contrary observations have been reported (228, 230) (see Table 17-2). The optimum levels of amylase additions have not yet been established and may be different for the two forms of the enzyme (90). The improvements recorded have been attributed to increasing availability of metabolic substrates (88, 89). They are most likely due to the shortening of the time necessary for capacitation to occur in the uterus, especially when inseminated in cows bred too late in estrus for normal capacitation to have occurred before ovulation (88, 91).

Beta glucuronidase, another of these enzymes, has resulted in important fertility increases when added to stored semen. The addition of 10, 30, or 150 Fishman Units of β -glucuronidase per milliliter of extender increased fertility

of semen in 1.0-1.5 units, respectively (90) (see Table 17-3). 150 units being compared 0 or 150 units of β -amylase per milliliter of extender. Responses with amylase at 28 and 112 days. When both enzymes were added, points over the control. At 112 days the β -amylase ($P < 0.05$).

60-90-day nonreturns (%)

Figure 17-4.
Pickett et al. (1970)

of semen in 1.0-milliliter ampules by 0.3, 1.2, and 5.9 percentage points, respectively (90) (see Table 17-3). The fertility response was almost linear, with 150 units being higher than either 10 or 30 units. O'Hagan et al. (229) compared 0 or 150 units of β -glucuronidase, with and without 10 micrograms of β -amylase per milliliter of semen packaged in 0.5-milliliter straws. The fertility responses with amylase were 1.9 and 1.8 percentage points above the controls at 28 and 112 days respectively, compared to 0.3 and 2.0 for β -glucuronidase. When both enzymes were added, fertility was improved 2.0 and 4.1 percentage points over the controls at 28 and 112 days after insemination, respectively. At 112 days there was a significant increase in fertility from the addition of β -amylase ($P < 0.1$), β -glucuronidase ($P < 0.05$) or both enzymes ($P < 0.001$).

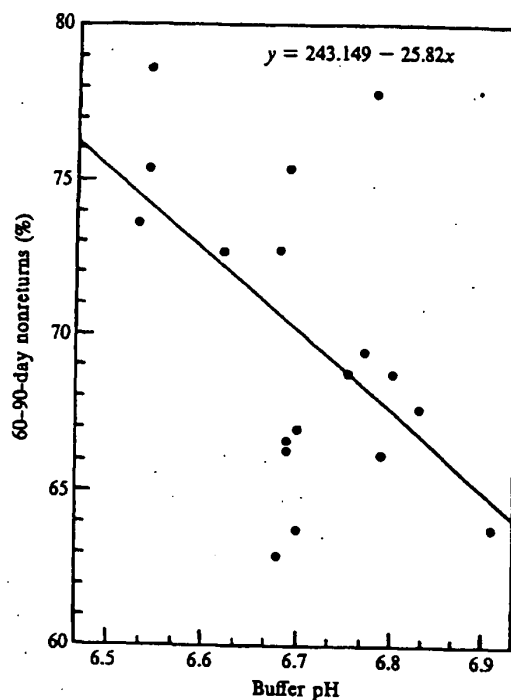


Figure 17-4. The effect of buffer pH on the fertility of liquid semen. [From Pickett et al. Conn. (Storrs) Agr. Exp. Sta. Bull. 373. 1962.]

Although the fertility response appears to be more favorably consistent to the addition of β -amylase than to the addition of β -glucuronidase, it has been postulated (229) that each additive contributes by hydrolyzing complex polysaccharides on the sperm-cell membrane. Contradictory fertility results have been obtained by others, however, (229, 246), so that final conclusions about the use of these enzymes, their amounts, and the best mode of action await further evidence.

Table 17-2. The effect of amylase on percent nonreturn^a using frozen semen.

Level of amylase (μ g/ml)	Type of amylase		Ref.
	Alpha	Beta	
0	68.1(4,294) ^b		(88)
1	69.0(3,807)	71.1(4,684)	
10	72.1(4,770)	70.7(4,336)	
0	67.1(1,414)		(87)
1 ^c	68.6(1,932)		
10	69.4(4,623)		
100	65.0(3,619)		
0	72.0(3,401)		(90)
10	74.3(1,504)		
20	74.2(2,057)		
0	72.9(307)		(228)
10	69.8(318)	70.2(323)	
0	73.4(6,152)		(89)
1	75.3(11,492)	75.5(11,329)	
10	75.1(12,084)	75.2(12,237)	
0 ^d	63.1(2,000) ^e		(229)
10 ^d		64.9(2,000) ^e	

^a60-90-day, except d.

^bNumber of first services in parentheses.

^cHighly purified, equal to about 10 μ g crude amylase.

^d112-day.

^eApproximate.

Table 17-3.

Lev.
β -glucuronidase
(μ /ml)
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0
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0
30
30

^a60-90-day.

^b112-day.

^cApproximate.

17-4 PREPARATION

17-4.1 In semen soon after collection, add an equal volume of glycerol. To terminate the freezing.

Benson subjected milliliter of cooled in 1:1 concentration.

Table 17-3. The effect of enzymes on percent nonreturn using frozen semen.

Level of enzyme		Inseminations	% NR	Ref.
β -glucuronidase	Amylase			
(μ /ml)	(μ g/ml)			
0	0	3,401	72.0 ^a	(90)
10	0	1,961	72.3	
30	0	1,912	73.2	
150	0	1,734	77.9	
0	0	2,000 ^c	63.1 ^b	(229)
150	0	2,000	65.1	
0	10	2,000	64.9	
150	10	2,000	67.2	
0	10	4,651	77.8 ^a	(246)
30	0	4,500	77.2	
30	10	4,337	76.1	

^a60-90-day.^b112-day.^cApproximate.

17-4 PREPARATION FOR FREEZING

17-4.1 Initial Extension Ratio. It is common practice to partially extend semen soon after collection to a constant spermatozoal number or to a standard volume for cooling to 5°C. After cooling, sufficient additional extender generally is added to provide twice the final number of sperm desired for insemination. The partially diluted semen is then glycerolated by the addition of an equal volume of extender that contains twice the final desired concentration of glycerol. The authors are aware of only one investigation designed to determine the influence of the initial extension rate on subsequent survival during freezing.

Benson et al. (92) evaluated the pre- and post-freeze motility of spermatozoa subjected to initial extensions to 1,000, 250, 40, or 20×10^6 spermatozoa per milliliter in either yolk-citrate or yolk-Tris extender. The semen was then cooled in four hours to 5°C, at which time all samples were extended to a final concentration of 20×10^6 spermatozoa per milliliter. The highest pre-freeze

and immediate post-freeze motility occurred at an extension to 250×10^6 and a trend toward lower motilities at higher extension rates was observed. A slightly greater rate may be possible with yolk-Tris extender.

In a subsequent experiment these authors (92) compared the motility of spermatozoa in similar extension rates after 0, 4, 8, and 12 weeks of storage at -196°C . No pre-freeze dilution effect was observed, but statistically significant differences were found favoring lower extension rates in immediate post-thaw motility and motility after four weeks of storage. Samples stored for longer than four weeks were not influenced appreciably by the initial extension ratio.

17-4.2 Incubation. The fertility of bull semen that is used immediately after collection is not as high as that of semen that has been kept for a period of time (see Chapter 19) (93, 94). At 4 to 5°C the period is a day or less, then fertility declines (95, 96, 97, 98). However, further evidence that fertility improves after storage at 5°C has been provided by studies on equilibration and spermatozoal maturation. Post-thaw motility and/or fertility was improved by increasing the interval at 5°C between collection and freezing (74, 75, 78, 99-103). Furthermore, it has been shown that four hours' cooling time was significantly superior ($P < 0.05$) to one hour (92). The changes in spermatozoa that occur during equilibration may correspond to those responsible for the increase in fertility of liquid semen observed from the day of collection to the following day.

Elliott et al. (104) showed that although post-freeze motility was somewhat depressed, fertility was improved after mixing antibiotics with neat semen, followed by dilution and incubation at 30°C for 30 minutes before cooling. An improvement ($P < 0.01$) in fertility of two low-fertility bulls was recently reported as a result of incubation at 26°C for 20 minutes (106) irrespective of antibiotics. Thus it is possible that the improvement in fertility shown in these studies (104, 105, 106) resulted from cellular maturation and not entirely from the additional time provided for antibiotics to reduce the number and pathogenicity of organisms (107).

17-4.3 Cold Shock. Cold shock occurs when bovine spermatozoa are subjected to sudden cooling. The magnitude of the effect depends on the terminal temperature, the rate of temperature reduction, the extension ratio, and the length of time that spermatozoa are exposed to the terminal temperature (66; 115). The consequences of cold shock are irreversible loss of motility, increase in the number of spermatozoa that are permeable to vital stains, and significant reductions in respiratory activity and glycolysis (66, 115-119, 124-128).

Blackshaw and Salisbury (117) have shown a significant uptake of sodium and calcium and a loss of potassium and lipid phosphorus in cold-shocked spermatozoa. Quinn and White (120) also reported an increase in sodium and

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calcium and a decrease in potassium and magnesium. It was further reported that dilution accentuated the cation movement of both cold-shocked and deep-frozen spermatozoa (120).

Other consequences of cold shock include coiled tails (121) and loss of lipid (19) and enzymes into the seminal plasma (122) (though a significant portion of glucose phosphate isomerase and lactic acid dehydrogenase are from protoplasmic droplets).

Epididymal spermatozoa are more resistant to cold shock than ejaculated sperm (123), and the loss of cold shock resistance seems to occur in the ampulla during ejaculation (124). Sperm in second ejaculates are more resistant to cold shock than sperm from first ejaculates (119), but this is probably true only when the interval between collections is longer than two days (125). Resistance to cold shock varies greatly among spermatozoa and is inherent in the individual spermatozoon (119, 123).

Cold shock can be prevented by cooling semen slowly in the presence of protective agents. The deleterious effects of cold shock are reduced by skim milk, casein, various milk extracts, and to some extent by bovine albumin, egg albumin, lecithin, egg yolk, and egg yolk extracts (116, 117, 118, 124). The protective mechanism must be one that stabilizes the cellular membrane thus reducing permeability. Dramatic changes take place in membrane permeability due to cold shock but more subtle changes may occur as well (126).

17-4.4 Cooling. It has generally been recommended that cooling of semen be commenced immediately after collection and extension, although, as stated above, an incubation period for raw or neat semen may be beneficial. In any event, semen should be added to an extender containing ingredients that will prevent cold shock and it should then be cooled slowly for maximum liveability of the cells.

VanDemark (108) and Foote and Bratton (109) have shown that the fertility of liquid semen was improved significantly ($P < 0.05$) by adding yolk-containing diluent to the semen before cooling. Despite this practice, rate of cooling can significantly affect fertility. Erickson (110) mixed semen and skim-milk extender at 32.2°C and then cooled split-samples to 3.3°C in either 110 minutes or four hours. Table 17-4 shows the fertility results of that study. The fertility of the samples cooled slowly (4 hours) was significantly ($P < 0.01$) higher than those cooled rapidly (1.8 hours).

In 1958, the National Association of Animal Breeders (NAAB) (111) found in a survey that, of the semen processors responding, 15 percent cooled semen in less than 1 hour; 52 percent in 1 to 1.5 hours; 10 percent in 1.5 to 2 hours; and only 23 percent allowed 2 or more hours for semen to cool.

Earlier laboratory experiments showed clearly that, for above-freezing storage, slow cooling was superior to rapid cooling in preserving motility and the capacity of sperm to metabolize substrates (112, 113, 114). An interaction was shown between temperature and cooling rate, indicating that a slower

Table 17-4. Effect on fertility, expressed as 30-40-day nonreturns,^a of two rates of cooling bull semen in heated skim milk plus antibiotics.

Variable	First services	% NR
Skim milk + antibiotics (cooled rapidly)	1.772	74.5
Skim milk + antibiotics (cooled slowly)	1.817	80.6
Skim milk + antibiotics + 10% glycerol (cooled slowly)	1.759	81.5

^aFrom Erickson (110). Unpublished results (1958) used by permission.

cooling rate was required for storage at 1°C than at 5°C (112). Similar results have been obtained (48, 92) for frozen semen. Post-thaw motility was maintained significantly longer in semen that was cooled 4 hours before freezing than in semen cooled only 1 hour (92). Similarly, use of live-dead staining techniques have shown that longer cooling times preserve the life of spermatozoa better than faster ones.

To our knowledge, no fertility studies have been conducted with frozen semen using cooling rate as a variable. The desire to complete semen processing and freezing on the day of collection seems to have resulted in a cooling rate of convenience rather than one based on sound experimental data. Although sufficient data are lacking for specific and precise recommendations, there is considerable danger in rushing the cooling process.

Figure 17-5 shows the equipment used in the laboratory at Colorado State University to obtain a slow (4 hours) cooling rate. It consists of a 600-milliliter nalgene beaker and a circular piece of masonite, 4½ inches in diameter, to which is glued a piece of cork ¾ inch thick and 3½ inches in diameter. A hole 1 inch in diameter is drilled through the masonite and cork. Tubes of several sizes are fitted with rubber rings to suspend the tube in the 600-milliliter beaker filled with 38°C water. Tube size varies to permit selection of a tube most suited for the volume of semen and extender.

A typical cooling rate, measured in the center of the test tube with a copper-constantin thermocouple, is presented in Figure 17-6. The rate of temperature reduction can be accelerated at any point along the curve by removing the tube and placing it in a test tube rack in the cold room.

Every AI unit should own and use a suitable, direct-reading instrument for measuring temperature with wire thermocouples (see Figure 17-7). The expense is minimal and could prevent some disastrous results. By placing the small thermocouple in the center of the diluted semen and conducting a few simple trial-and-error experiments, the desired cooling rate can be established for any set of conditions. Furthermore, the temperatures of the refrigerators,

Chapter 17:

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frequent intervals.

17-4.5 Glycerol Ac

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have concluded tha
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Fig.



Figure 17-5.

various locations within cold rooms, and waterbaths should be checked at frequent intervals.

17-4.5 Glycerol Addition.

Temperature of Glycerol Addition. Polge (127) reported that spermatozoan revival after freezing was greater when glycerol was added at 5°C than when it was added at 20°C. Miller and VanDemark (43) obtained a slightly higher percentage of motile spermatozoa when glycerol was added at 4.5°C than at 10°C and both of these were superior to addition at 15.5°C ($P < 0.01$). Others have concluded that spermatozoa were damaged upon exposure to glycerol at temperatures above 5°C (53, 128, 129) and found that the damage to



Figure 17-5. Equipment that will permit slow cooling of semen.

spermatozoa exposed to glycerol at 32°C was more pronounced as the length of glycerol exposure at this temperature increased (130).

Other investigators (76, 131, 132) have not observed a depression in post-thaw motility following glycerolization at temperatures above 5°C. In one investigation there were no differences in 75-day NRs for spermatozoa glycerolated at 5, 10, 20, or 35°C (132).

The damaging effects of exposure to glycerol at higher temperatures are at least partially reversible upon subsequent equilibration at 5°C (43, 130). It is likely that survival would be most severely depressed in samples glycerolated at higher temperatures when equilibration time was also shortened.

Loss of the enzyme glutamic-oxalacetic-transaminase (GOT) was greater from bull spermatozoa glycerolated in yolk-citrate at 37°C than at 5°C. The rate of release was no different after 4 hours of equilibration at those temperatures (133). With a Tris-yolk extender and 3 hours' equilibration there was no depression of fertility by addition of the glycerol at 35°C (78).

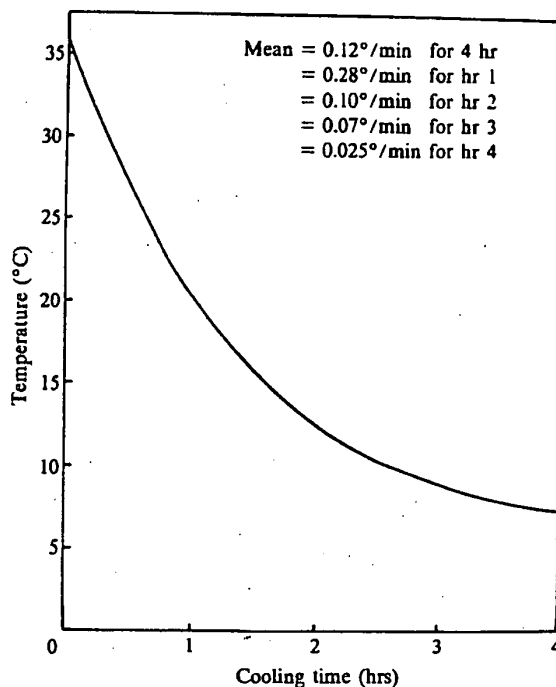


Figure 17-6. A typical semen cooling rate at the center of the test tube depicted in Figure 17-5.

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Rate of Glycerol Addition. Post-thaw motilities of 38 and 51 percent were observed when glycerol was added in 1 or in 3 steps at 20-minute intervals (53), respectively. In a subsequent experiment no significant differences were found between 4 and 16 equally spaced glycerol additions during a 1- or 2-hour period. An advantage of stepwise addition of glycerol to bull semen was observed, but no more than 4 additions were necessary.

In contrast, no differences were found in the post-thaw motility of spermatozoa glycerolized at 5°C in 5 equal additions at 6- or 12-minute intervals, in 3 equal additions at 10-minute intervals, in a single addition (134) or an experiment with glycerol addition in 1, 3, or 5 equal portions (43). The fertility of spermatozoa frozen after glycerolization at 5°C in either 1 or 4 steps immediately after cooling was compared with 1 step 5 hours after cooling (1 hour

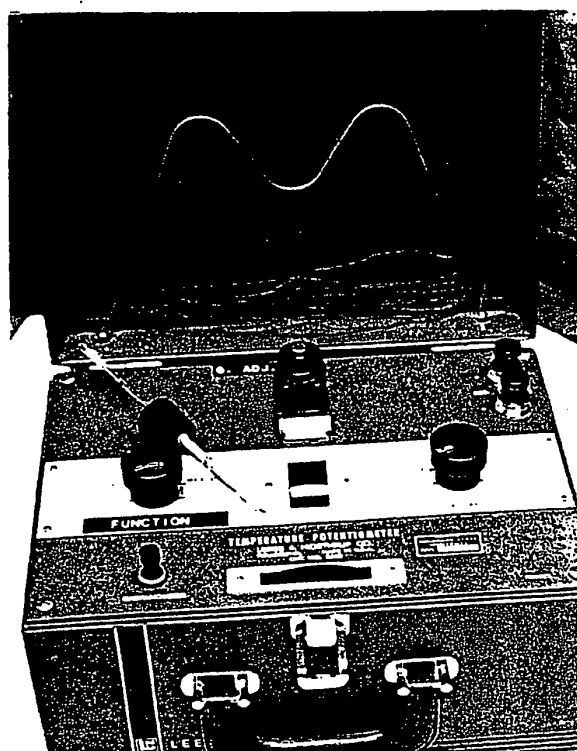


Figure 17-7. A potentiometer capable of measuring temperature by means of wire thermocouples.

before freezing). Fertility was not significantly influenced by the method of glycerol addition; it was similar for semen frozen in straws when samples were glycerolated either slowly, over 2 hours, or quickly (135).

Under some conditions the stepwise addition of glycerol to bull semen may enhance post-thaw motility; the advantage has generally been small, however, and may not be reflected in increased fertility (78, 135).

It has also been reported (136) that the liveability of spermatozoa in liquid semen was enhanced by adding glycerol stepwise in 20, 30, and 50 percent portions at 10-minute intervals, as compared to addition in a single step. Drop-wise glycerolization has been employed successfully for both liquid (136) and frozen semen programs.

Shannon in New Zealand (137) has described a novel approach to the bulk freezing of semen that may be applicable to the seasonal breeding of beef cattle in the United States (146). Semen was initially diluted about 1:1 for cooling to 5°C, followed by dilution to a concentration of 600×10^6 sperm/ml without glycerol. The extended semen was then placed in dialysis tubing about 5 inches long and 0.25 inches in diameter, and suspended in extender that contained glycerol. This allowed the exchange of glycerol and metabolic end-products. Following a period of equilibration, the material in the dialysis sack was frozen. By freezing semen in bulk at a high sperm concentration, efficient utilization of labor and storage space was achieved. This technique was well suited to the seasonal breeding of dairy cattle in New Zealand.

17-4.6 Equilibration. Investigators (35, 74, 75, 99-103, 138) have demonstrated that post-thaw motility and fertility of bovine spermatozoa are both enhanced by extending the interval between adding the glycerol and beginning freezing. This stage of semen processing has been called the "glycerol equilibration" period. The results of several fertility trials designed to determine the optimal glycerol equilibration period have been summarized in Table 17-5. These studies clearly established the beneficial influence on the fertility of bull spermatozoa of an equilibration period of several hours.

Experiments have been conducted in which equilibration times have been varied at 5°C both in the presence of glycerol and without glycerol (53, 78, 101, 139, 140). The goal has been to determine whether it is spermatozoan-glycerol interaction or a process of sperm-cell maturation. A number of investigators (35, 75, 78, 138) have found evidence that lengthy glycerol equilibration per se is not as essential as the time in storage at 5°C before freezing. Jondet's (141) experiments using straws as containers for frozen semen suggest the same conclusion.

It is clear that additional fertility trials with the different methods of freezing are desirable, but on the basis of existing information 4 to 18 hours of storage at 5°C before freezing are recommended; and it appears that glycerol can be added at any time during this period without affecting fertility.

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Table 17-5. The influence of glycerol equilibration on non-return rates.

Equilibration time	No. services	% NR	Ref.
0.5 hr	449	56.2 ^a	(99)
18 hr	450	63.9	
30-50 min	334	71.8 ^b	(100)
overnight	330	78.1	
0.5-1.0 hr	864	54.4 ^c	(101)
18 hr	864	61.4	
4 hr	996	63.4 ^d	(102)
8 hr	1,026	65.2	
12 hr	1,012	67.8	
2 hr	2,256	66.5 ^e	(103)
18 hr	2,176	71.3	

^a30-day NR.

^d75-day NR.

^b31-day NR.

^e60-90-day NR.

^c3-month NR.

17-4.7 Sperm Numbers. The demand for semen from genetically superior sires has made it necessary that a minimum number of frozen sperm cells be established for maximum fertility of each insemination (142-154). Fertility is the single most important consideration in AI and no fewer sperm should be used than are necessary. One problem in recommending a minimum number is that new extenders and new methods of processing, freezing, and packaging make continual reevaluations necessary.

Table 17-6 shows the influence that the number of spermatozoa per insemination has on the fertility of semen frozen in 1.0-milliliter ampules. Since only one study (231) resulted in statistically significant differences, the lowest number of spermatozoa could logically be selected in all except that study. In all except one of the seven studies, however, mean fertility was higher with the highest number. Thus for maximum reproductive efficiency, the higher numbers of spermatozoa presented in Table 17-6 should be used.

Experiments illustrated in Figures 17-8 and 17-9 (151, 186) show that the number of spermatozoa per insemination is somewhat higher for the bull with

lower than average fertility. Thus the fertility level of each bull in a breeding operation must be considered before the absolute number of spermatozoa for each insemination can be established.

Table 17-7 summarizes the results of experiments on the number of spermatozoa necessary in extended semen that is to be packaged and distributed in straws (135, 147, 148, 149, 153, 189, 232-234, 235). These studies have also shown differences among bulls. In another study (153) the fertility difference between "good" and "poor" inseminators decreased as sperm numbers increased.

17-4.8 Packaging: Ampules, Straws, Pellets. At first, bull semen was packaged for freezing in glass ampules. Then a process for freezing extended semen in straws was developed, and more recently semen has been frozen in pellets. Satisfactory conception rates have been reported with all three systems (247, 248).

Danish investigators developed the idea of packaging and freezing extended semen in straws and were also responsible for much of the other earlier work. Soda straws were used in the first studies. French workers Cassou (156) and Jondet (157) have refined the technique. The French straws are plastic containers 113 millimeters long. They vary in diameter from 2.0 millimeters (0.25 milliliter) to 4.2 millimeters (1.2 milliliters), with an intermediate diameter of 2.8 millimeters (0.5 milliliter). More recently, groups in Canada, West Germany, and the United States have developed systems using straws of various sizes (see Figure 17-10). Macpherson and Penner (155) have reviewed the history of this technique and have described a modified method developed in Canada.

In the French and Canadian techniques, one end of the straw is plugged with a powder of polyvinyl alcohol that forms a seal when it comes into contact with an aqueous solution, the semen. The other end is ultrasonically or electrostatically sealed after filling. West German and U.S. straws are sealed with nonabsorbable plugs (see Figure 17-10).

The system has now been automated and its popularity is likely to increase. The straw system requires less cold storage space: Field units will accommodate at least twice as many straws as ampules; and central storage units will hold three to five times as many (158). There might also be some advantage with regard to fertility (see Table 17-8) (247, 248).

The technique of freezing semen in pellets was originally developed by Japanese workers (44, 163) and has not been widely adopted by the AI industry in the United States or Western Europe. The system has not been automated, and permanent labeling of the pellets has proven difficult. Furthermore, there is the possibility of contamination (248) by bacteria in the process. To freeze semen by the pellet method, small depressions are made in a block of solid CO₂ to accommodate the desired volume of semen. Volumes of 0.05 to 0.2 milliliter are placed in the depressions on the dry ice (see Figure 7-11), where the semen is allowed to remain for about 10 minutes before transfer to liquid nitrogen storage.

Table 17-6. The influence of number of spermatozoa per insemination on the fertility of semen frozen in 1.0-milliliter ampules.

Spermatozoa (10 ⁶)	Inseminations	% NR	Ref.
13.3 ^a	490	61.0 ^b	(84)
20.0	490	63.3	
30.0	489	61.5	
20.0 ^c	5,607	71.7 ^d	(146)
30.0	5,638	72.2	
16.0 ^c	1,977	72.8 ^d	(78)
24.0	2,103	74.6	
16.0 ^c	5,916	73.1 ^d	(78)
24.0	5,831	73.2	
12.0 ^c	13,939	70.4 ^d	(78)
24.0	13,060	71.6	
12.0 ^a	— ^e	70.6 ^d	(151)
24.0	—	72.0	
35.0	—	73.1	
10.0 ^c	1,576	61.1 ^d	(231)
15.0	1,488	61.5	
20.0	1,539	69.5	

^aTotal spermatozoa.

^b3-month.

^cMotile spermatozoa before freezing.

^d60-90-day.

^e20,815 cows were utilized for the experiment.

Table 17-7. Influence of number of spermatozoa per insemination on the fertility of semen frozen in straws.

Spermatozoa (10 ⁶)	Inseminations	% NR	Ref.
10 ^a	557	61.6	(232)
20	527	61.9	
30	565	65.7	
5 ^a	624	55.6	(232)
10	622	58.7	
20	638	61.0	
10 ^a	565	79.5	(147)
20	527	78.6	
30	557	78.6	
5 ^a	532	76.5	(147)
10	533	74.5	
20	550	77.3	
20 ^a	2,665	58.4	(233)
30	2,888	60.2	
8 ^b	2,648	69.7	(149)
12	2,551	73.3	
.375 ^c	805	49.9	(148)
.75	816	57.4	
3	765	65.8	
14	836	71.8	
5 ^a	993	74.0	(234)
10	998	79.5	
15	990	76.7	
20	995	79.2	

^aTotal spermatozoa.

^bMotile spermatozoa before freezing.

^cLive spermatozoa after thawing.

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17-5 FREEZING AN

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Spermatozoan concentrations in the pellets are about 10 times higher per milliliter of extended semen than in ampules, and the pellets therefore use far less storage space. Pellets are thawed rapidly in enough warm extender (163) to provide the right volume and the proper number of spermatozoa for each insemination.

The choice of packaging system should depend upon conception rate, economics, and convenience, in that order.

17-5 FREEZING AND THAWING

17-5.1 Freezing Rates. In early studies, ampules of semen were frozen in a dry ice-alcohol bath. Cooling rates of 0.8 to 3°C per minute from 5 to -15°C, and 3 to 5°C per minute from -15 to -79°C, were maintained by controlling the rate at which dry ice was added to the bath. Slow freezing rates were considered essential until Luyet and Keane (164) froze semen by transferring

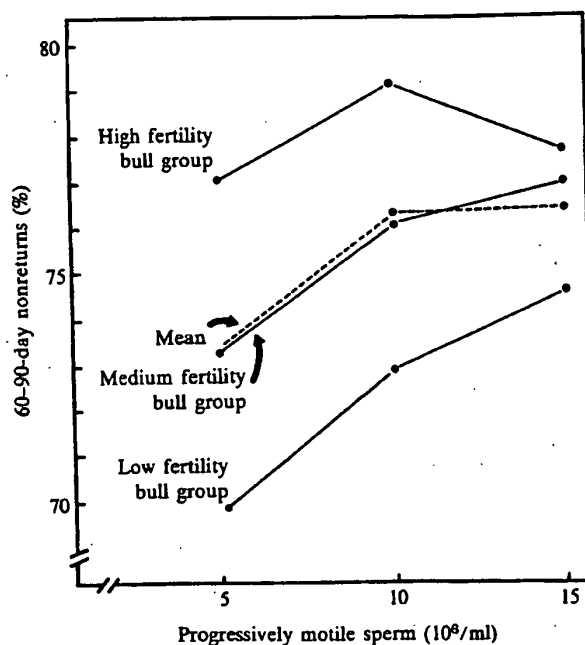


Figure 17-8. Nonreturn rate as affected by motile spermatozoan concentrations and fertility level of Holstein bulls. [From Sullivan. Proc. 3rd NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 36-43. 1970.]

ampules abruptly from 0°C to an alcohol bath at temperatures from -20 to -50°C, where it was held for five minutes and then transferred abruptly to liquid nitrogen. When the intermediate temperature was -20°C virtually none of the cells survived freezing. In contrast, an intermediate temperature of -27°C provided a high rate of survival. Thus the existence was established of a critical temperature range in which rapid cooling was detrimental to spermatozoan survival. This finding was supported by a number of investigations in which conventional freezing rates were employed (165, 166) and was consistent with the report (167) of an optimal cooling rate of 4 to 20°C per minute between -10 and -30°C (see Figure 17-12).

In contrast, other workers (168) found that semen could be transferred directly to the bath at -20 and -40°C for subsequent freezing at the normal

rate. It was apparent that the use of intermediate temperatures and -40°C. The pellets (32, 163). With these techniques, a high rate of survival was achieved at -196°C (see Figure 17-13) using a variety of techniques.

The involvement of the spermatozoa in the evolution of the process to the point where the spermatozoa are in liquid nitrogen contains and they eliminate the system depends on the freezing curves. Figure 17-14. The spermatozoa occupy different positions in the spermatozoa can be

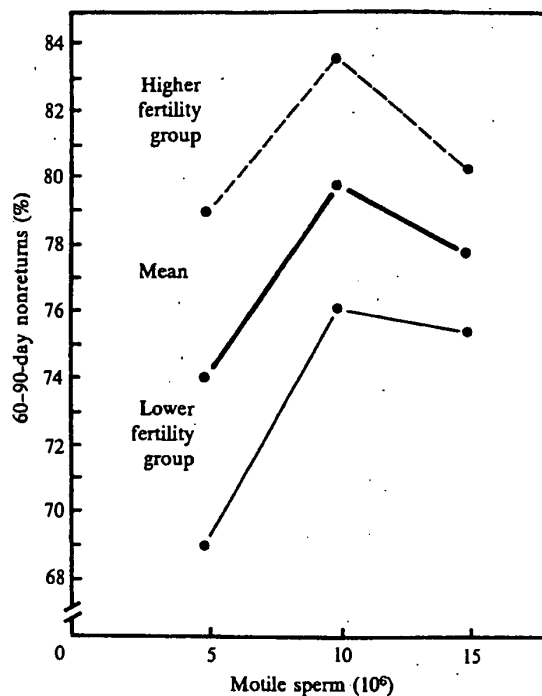


Figure 17-9. Nonreturn rate of dairy cows as affected by motile spermatozoa concentration and fertility level of semen from Angus and Hereford bulls. [From Sullivan. Proc. 3rd Tech. Conf. Artif. Insem. and Reprod. NAAB. pp. 36-43. 1970.]

rate. It was apparent that spermatozoa could be cooled rapidly between 5°C and -40°C. This was supported by the successful freezing of spermatozoa in pellets (32, 163), films (169), and by direct placement into liquid nitrogen (32). With these techniques spermatozoa are cooled rapidly from 5°C to -79° or -196°C (see Figure 17-13) (170). Spermatozoa may be frozen successfully using a variety of rates and methods.

The inconvenience of manually regulating the cooling rate has led to the evolution of programmed instrumentation techniques and then simplification to the point where the semen is now frozen in the nitrogen vapor of a liquid nitrogen container (171-174). These techniques are simple and convenient and they eliminate the need for costly automation. The freezing rate in such a system depends upon the ampule's position within the nitrogen vapor. Typical freezing curves for ampules frozen in liquid nitrogen vapor are presented in Figure 17-14. The comparable survival rates of spermatozoa in ampules that occupy different positions within the vapor supports the contention that spermatozoa can withstand a wide range of freezing rates.

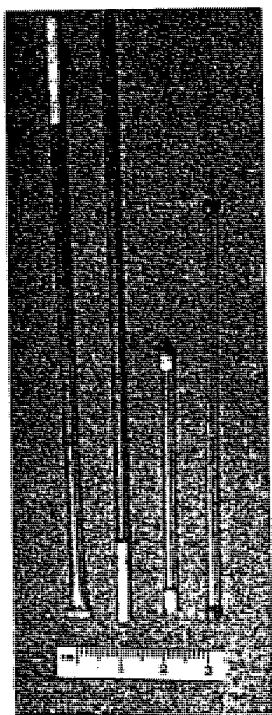


Figure 17-10. Various types and sizes of straws currently in use. From left to right: Canadian, French, U.S., and German straws.

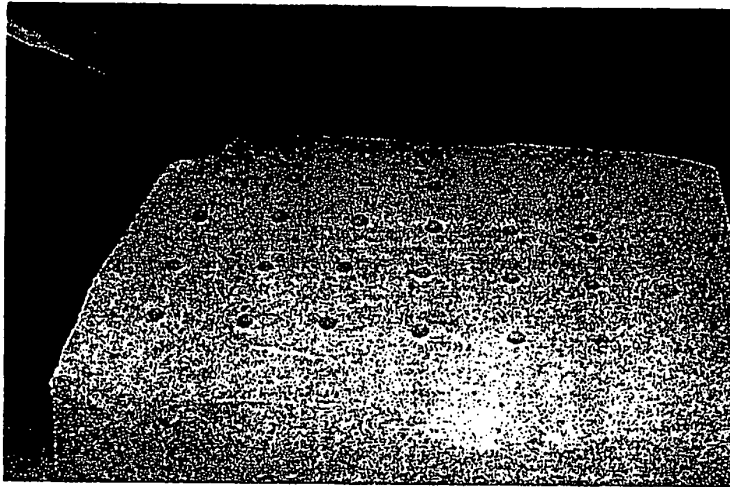


Figure 17-11. Semen being frozen by the pellet method.

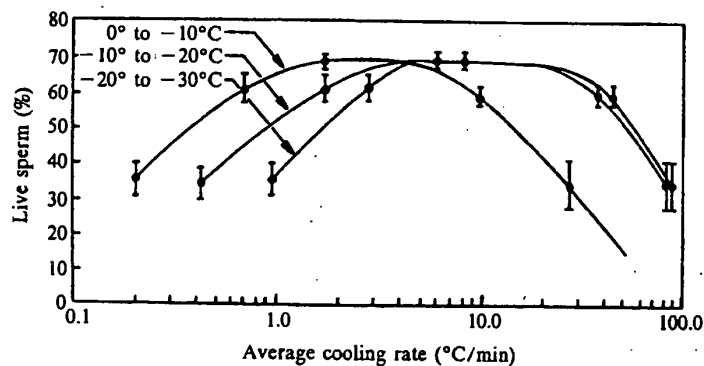


Figure 17-12. The optimum range of cooling rates between -10° and -30°C . [From Bruemmer et al. *J. Cell and Comp. Physiol.* 62:113-118, 1963.]

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17-5.2 Storage Time
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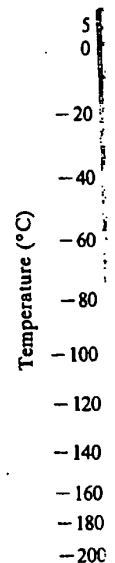


Figure 17-13. C
left to right repre
at -79° , -100°
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If the current popularity of the straw method of packaging continues, the use of vapor freezing methods will become more extensive.

17-5.2 Storage Time and Temperature. The cost of storing material at temperatures below freezing increases as the temperature decreases. Thus for economical reasons it is necessary to determine the temperature that will provide extended storage, minimum expense, and minimum deterioration in spermatozoan quality. VanDemark et al. (37) have shown (see Figure 17-15) that storage temperature has a dramatic effect on sperm motility after storage.

A number of detailed experiments have been conducted to elucidate the details of the effects of storage temperature and duration on the survival and fertility of frozen bull spermatozoa. The results show not only the effects of the main factors already discussed but important interactions among these factors. In general the data indicate that the lower the storage temperature the longer motility and fertility can be maintained. To ensure this, however, it is

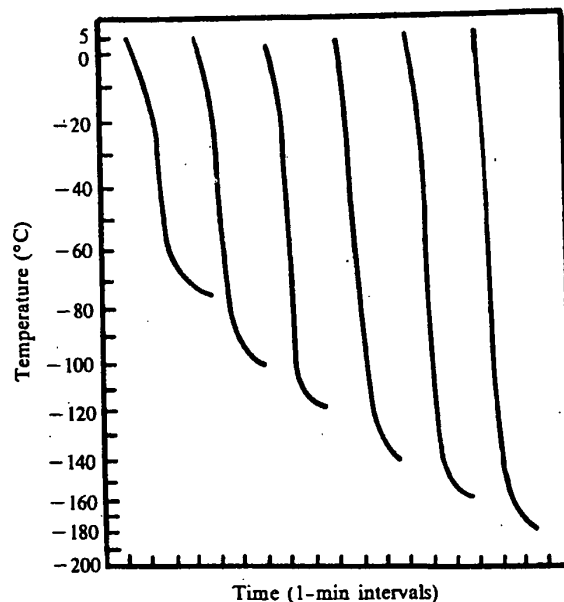


Figure 17-13. Cooling rates at the center of 0.3-milliliter pellets. Curves from left to right represent freezing on dry ice (-79°C), and on a stainless steel plate at -79° , -100° , -110° , -140° , -160° , and -180°C , respectively. [From Salamon. *Aust. J. Biol. Sci.* 23:459-468. 1970.]

necessary to use the procedures, timing sequence, and other techniques that are optimum for that lower holding temperature. In one experiment (175) using split ejaculate samples, the motility of the spermatozoa in semen frozen to -79°C declined more rapidly during incubation after thawing than the spermatozoa in semen that had not been frozen. When a thrice-repeated freezing-thawing stress was applied to semen when it was first frozen to either -79°C or -196°C no differences were observed in the resistance of the contained spermatozoa to this stress at either temperature. When the semen was stored for 6 months at these temperatures, however, the spermatozoa kept at -196°C withstood the repeated stress better than those kept at -79°C (176). Similarly, when semen was exposed to ambient room temperature for short intervals after 0 to 120 days' storage, spermatozoa in liquid nitrogen were more resistant than those from the same ejaculate frozen in dry ice/alcohol (177). Greater deterioration in motility and metabolic activity of spermatozoa occurs at -79°C storage than at -196°C regardless of the storage interval (178, 179, 180).

Semen transferred from -196°C survived better on subsequent storage at -79°C after holding at -196°C for 7 or 10 days rather than for 3 days (182).

Investigations involving temperatures of -79 , -92 , -96 , and -196°C show an advantage for temperatures below -79°C (80, 181) for spermatozoan survival and motility recovery. Spermatozoa in milk extenders, however, appear to require a lower holding temperature than yolk-citrate extender for optimum motility recovery after several months of storage (80).

Some investigators have reported a gradual improvement in the post-thaw recovery of motility after 3 weeks' storage at -196°C (179) and for as long as 24 weeks at -96°C , suggesting that, over time, spermatozoa make a significant adjustment to the conditions of storage (181). More recent studies (183, 184) show that fertility increases for a time immediately after freezing. A similar increase has been observed at the beginning of storage at temperatures above freezing. Whether the fertility of frozen semen in commercial AI will increase with storage depends largely on the procedures used in processing and freezing. Fertility is not likely to increase if, for example, the semen has been subjected to a relatively long incubation or equilibration after cooling immediately upon collection and before freezing.

Studies on the effects of temperature on rates of chemical change and the effects of storage indicate that fertility will be maintained longer at the lowest practical storage temperature (-196°C) than at higher temperatures. Considering the expected temperature control of metabolic turnover from body temperature to -196° , no system of semen storage has produced results comparable to those in which rabbit spermatozoa in the ligated epididymis were held in the scrotum at a few degrees below body temperature (see Section 8-7.4). Based on a temperature coefficient of 2, which would halve the metabolic activity each drop of 10°C the 38-day period found by Hammond and

Asdell (251) for fertility be comparable to a fertility in cattle sperm has ever been.

Mixner (154) has years), that bull sperm and at -196°C after term storage tests with a conception rate of decrease of 0.8 percent stored in glass amp

Table 17-8. Effect

Straws	
% NR	Inse
67.2	
73.0	
70.4	
70.7	
63.7	
71.1	
72.0	
67.7	
67.5	
61.1	
69.0	
71.0	
74.3	
71.5	
73.2 ^a	
70.7	
74.9	

^aPercent pregnant by

^bFirst and repeat serv

Asdell (251) for fertility maintenance in the ligated rabbit epididymis would be comparable to a hundred years or so at -196°C . Theoretically, optimum fertility in cattle spermatozoa can be maintained far longer at -196°C than it has ever been.

Mixner (154) has shown, with a limited number of services (626 in the 12 years), that bull spermatozoa remain fertile when stored at -79°C for 8 years and at -196°C after that for up to 12 years. After performing similar long term storage tests with a total of 1310 inseminations Stewart (185) has reported a conception rate of 58 percent after 9 years. This represents an average yearly decrease of 0.8 percent NR. The report by Salisbury (183, 186) for bull semen stored in glass ampules at -79 to -88°C refers to all semen used by one AI

Table 17-8. Effect on fertility of packaging semen in straws or ampules.

Packaging system				
Straws		Ampules		Ref.
% NR	Inseminations	% NR	Inseminations	
67.2	—	64.3	—	(236)
73.0	78	73.9	69	(237)
70.4	1,462	67.7	1,909	(161)
70.7	888	73.8	725	(238)
63.7	—	57.9	—	(239)
71.1	305	69.5	240	(159)
72.0	150	67.8	158	(240)
67.7	176,293	69.1	161,151	(241)
67.5	7,380	67.6	7,188	(160)
61.1	3,915	65.5	3,847	(160)
69.0	—	67.5	—	(242)
71.0	599	67.0	625	(243)
74.3	354	73.2	381	(243)
71.5	5,544	70.3	5,637	(243)
73.2 ^a	395 ^b	54.3 ^a	383 ^b	(244)
70.7	1,407	72.7	1,448	(245)
74.9	7,592	72.8	6,714	(162)

^aPercent pregnant by rectal palpation.

^bFirst and repeat services.

organization for inseminations during 1962 and for all semen collected in 1962 and used either in that year or in 1963. A total of 216,222 first service inseminations were involved. For a major organization at that time it was a very precise study of fertility expectation and of seasonal influences on fertility; but it was a less precise estimate of the potential duration of fertility for selected semen. Fertility peaked after 2 months of storage at -79°C , and stayed at a maximum for about 4 months, after which it decreased at the rate of 5 to 6 percent NR a month. When semen is transferred from liquid nitrogen to dry ice-alcohol the ability of the spermatozoa to recover motility is depressed (178, 179, 182). This ability lasts longer in liquid nitrogen (80, 176, 177, 178, 180, 182). Direct comparisons of fertility after storage at the two temperatures for less than 6 months are slightly in favor of the lower temperature (145, 182, 185) (see Table 17-9), but no long term comparisons have been made of fertility duration using samples of the same semen stored at the two temperatures. Salisbury's study (183) dealt with the fertility level found for the same AI unit after a shift to semen freezing and storage under liquid nitrogen by

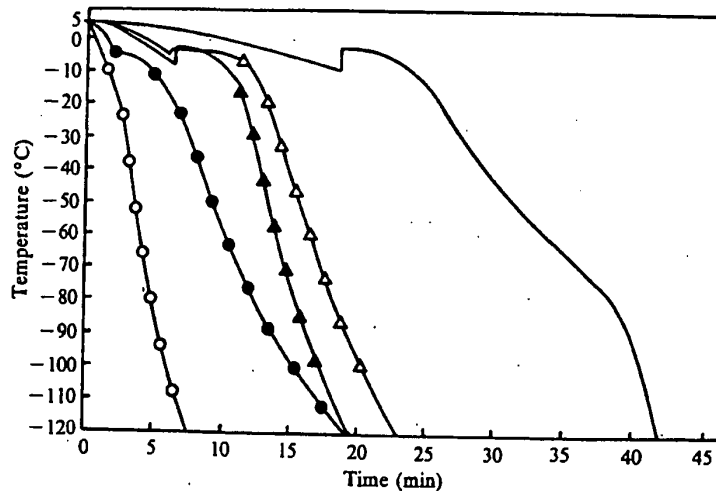


Figure 17-14. Representative intra-ampule cooling rates for semen frozen in an automatic freezer and in liquid nitrogen vapor. The various lines represent the following: —, control (automatic freezer); Δ , top ampule in center of cardboard box (vapor frozen); $\blacktriangle$, third ampule from top in center of box (vapor frozen); $\bullet$, third ampule from top in outside corner of box (vapor frozen); and $\circ$, bottom ampule in center of box (vapor frozen). [From Clegg et al. Univ. of Conn. (Storrs) Agr. Exp. Sta. Res. Rep. 5. 1965.]

the inseminator technicians with weekly shipments out of central storage. The results for 196,448 first service inseminations show an increase in fertility for the first 4 months of storage at -196°C , after which fertility peaked for the next 8 months and then decreased during the second year of storage. Clegg and Pickett (187) studied 21,488 inseminations with semen stored at -196°C . They found no decrease in fertility over 2 years of storage. Foote (191), who obtained the same results, also found no evidence of important seasonal variations in fertility such as those that had been shown in the Illinois studies (183, 186). Fertility may last longer under liquid nitrogen if the storage conditions, including the actual semen temperature, are kept constant.

In studying semen stored in straws at -196°C Cassou (189) found no differences in fertility between 1 and 3 months (69.8 percent NR on 9,053 first services); between 4 and 6 months (68.4 percent NR on 11,472 first services); or between 7 and 66 months (69.1 percent NR on 83,843 first services). Ström (188) made a similar study of semen frozen in pellets and held in liquid nitrogen. A total of 63,863 cows were inseminated. He found no evidence of a change in fertility over a mean storage period of 44 weeks.

The latest data available on the questions raised by these studies are reported in a recently completed statistical analysis at the Illinois Agricultural Experiment Station (252). This analysis was performed on data provided by

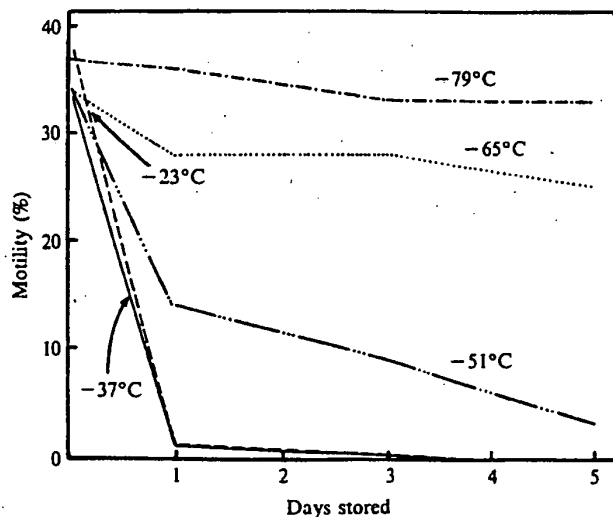


Figure 17-15. The effect of storage temperature upon post-thaw motility. [VanDemark et al. Illinois Agr. Exp. Sta. Bull. 621. 1957.]

Table 17-9. Effect of storage temperature on nonreturn rate.

<i>Storage temperature</i>					
<i>−79°C</i>		<i>−196°C</i>		<i>Difference</i>	<i>Ref.</i>
<i>First services</i>	<i>% NR</i>	<i>First services</i>	<i>% NR</i>		
463	69.3	495	71.9	2.6	(182) ^a
1,300	58.4	1,251	60.1	1.7	(185) ^b
4,791	68.4	4,951	71.1	2.7 ^d	(144) ^c

^a75-day NR.^b112-day NR.^c60-90-day NR.^dP < 0.01.

the Michigan Animal Breeders Cooperative on 555,449 first service inseminations made with semen stored in ampules at -196°C from 1965 to 1970. Some of the semen was used 5 years after collection. The day of seminal collection and the day of insemination were recorded for all inseminations. The data show the fertility responses to length of storage were significantly different among ejaculates both from different bulls and from the same bull. Fertility increased with time after collection and freezing (the use of semen on 5th to 15th day after freezing tended to yield the lowest 180-day NR rate). In one separate analysis of 97,586 inseminations using semen from 123 bulls maximum fertility (180-day NR) occurred after 810 days or about 2.2 years of storage. In a 2-way analysis of variance of 108,311 inseminations using semen from 133 bulls maximum fertility occurred when semen had been stored 1260 days or about 3.4 years. In neither case was there a statistically significant decrease in fertility with time up to 1260 days, though the variability did increase greatly. In each case, however, these samples were selected to test, as well as possible, the duration of fertility under nitrogen storage. When a regression analysis was calculated, using semen one year old or less and involving 83,198 inseminations from the semen of 122 bulls, the maximum fertility occurred for semen that was 255 days old. This fertility level stabilized for a time and then declined. These analyses indicate that, with proper selection of bulls and ejaculates and proper handling, some semen can maintain optimum fertility for relatively long periods—up to 3.4 years in one study.

17-5.3 Handling Techniques. Until recently, most cows in commercial AI programs were bred by full-time, closely supervised technicians. The sale of semen directly to the herd owner, with insemination to be performed by him

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or by a farm employee, has posed an entirely new set of problems. This is particularly true with the AI of beef cattle, in which an inseminator often breeds a large number of animals on a seasonal basis during a short period of time. This often requires an annual relearning of skills, often with little or no supervision. It is therefore important for the inseminator to review the proper methods of semen handling periodically throughout the year (190). Many of the fertility problems associated with the use of frozen semen are due to improper handling or deposition of the semen.

In many cases, the first mistake comes when the semen is received at the farm or ranch and must be transferred from one storage unit to another. This should take place out of the wind and sunlight. Wind increases the rate at which the temperature of the ampule rises by removing the "layer of cold" that surrounds the ampule. The effect of sunlight on frozen semen is not known, but it is not likely to be beneficial and it could actually be harmful.

Frozen semen is further exposed to elevated temperatures when the semen is removed from storage tanks, when they are opened as inventories are taken, etc. The amount the temperature rises is determined by:

1. length of time exposed.
2. ambient temperature.
3. air circulation,
4. intensity of solar radiation,
5. nitrogen level in the tank, and
6. the height to which the canister is raised above the liquid nitrogen level.

When semen is transferred the exchange should be made quickly into tanks previously filled with nitrogen. It is essential that semen is handled properly, which involves careful temperature control more than anything else, in order to prevent fertility reduction. It is reasonably clear from the work reported by Sullivan (150) (see Figure 17-16) that, as sperm numbers are reduced to obtain maximum use of each superior bull, the storage temperature, exposure times, and so on will become more critical. It has been shown that exposing frozen semen in the neck of a liquid nitrogen tank generated no significant decrease in motility unless the exposure was relatively long (192). The fertility of fluid semen, however, decreases much more rapidly than motility (79) and the same is probably true of frozen semen. A fairly high correlation has been shown (177) between fertility and loss of motility due to exposure of ampules of frozen semen to ambient temperatures. Thus it is extremely important to minimize the number and times of exposures (see Figure 17-17). It is much more harmful to expose ampules of semen to elevated temperatures uninterruptedly for one minute than to expose them for two minutes in 10-second periods, particularly if the ampules are returned to -196°C between the 10-second exposures.

17-5.4 Thawing. It is difficult, if not impossible, to separate the effects of thawing from those of freezing. Theoretically, the faster a specimen is frozen the more rapidly it should be thawed for optimal survival (193). Furthermore, the results obtained from thawing studies may be influenced by other events (43, 128, 167, 193, 194). Interrelationships have been shown between thawing and freezing rates (167) and extenders (128) and other factors, such as glycerol concentrations, may contribute to some of the discrepancies in the literature (194).

Rate of thawing significantly affects the survival of spermatozoa that have been frozen (43, 128, 134, 159, 167, 194). The rate at which thawing occurs depends on the size and shape of the container (ampule, pellet, straw) in which the semen is stored, the composition of the container (plastic, glass), the thawing medium (water, air), the temperature imposed, and the type of thawing system (195).

Thawing in Glass Ampules. It has been reported that thawing bovine semen in ampules in water maintained at 38 to 40°C resulted in superior sperm survival

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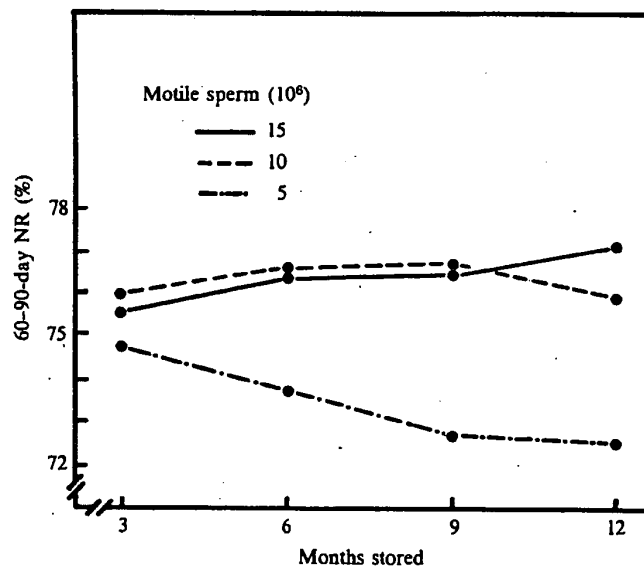


Figure 17-16. The influence of sperm numbers on the fertility of semen stored for up to one year. [Sullivan. Proc. 3rd NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 36-43. 1970.]

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as compared to thawing at lower temperatures (128, 167). Müller and Van-Demark (43), however, using 5-milliliter plastic ampules containing 1 milliliter of extended semen, observed significantly greater motility in samples thawed at 5°C than in those thawed at 38°C; others (134, 194, 196, 197) found no difference in survival between spermatozoa water-thawed at 38 to 40°C and those thawed at 5°C. In general, thawing in air or water at intermediate temperatures of 15 to 22°C resulted in decreased survival (134, 142, 194, 198). It is exceedingly difficult to explain why an intermediate thawing rate is inferior to a slower rate, since it has been postulated (127, 128) that thawing time between -15 and -30°C should be as rapid as possible. Although this may be expected on a theoretical basis, the critical range for freezing may not correspond to a critical range for thawing.

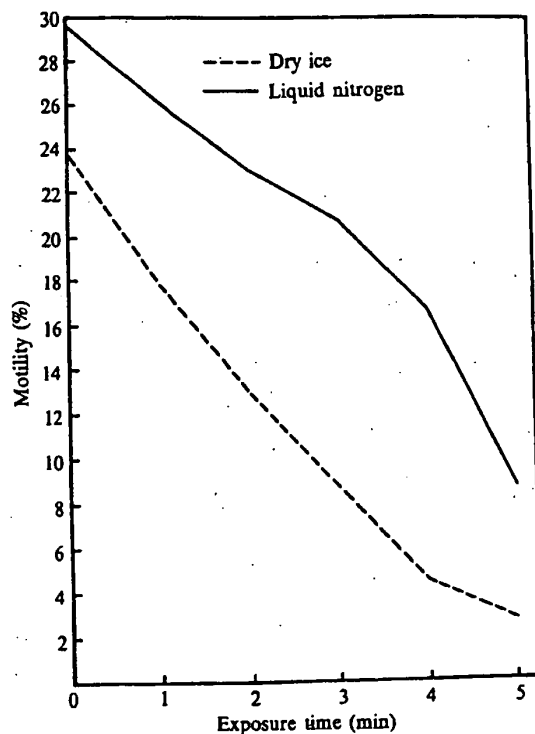


Figure 17-17. The effect of exposing ampules of frozen semen to ambient temperature on percent motility. [From Pickett. *A.I. Digest* 19(2):8-23, 1971.]

The end fertility level is the ultimate test of superiority of any semen handling method. Unfortunately such data on thawing rates are limited. Arnott (142) conducted two studies comparing semen thawed in water at 15 and 30°C, and at 4 and 15°C. A significant difference in 60-90-day percent NR was found in favor of 15°C in the first study, and a 2.0 percent NR difference in favor of thawing at 4°C in the latter study. These latter results agree with those of Pickett et al. (194), who found a difference of 1.26 percentage points in NR between 1 and 15°C thawing in favor of the colder temperature. Dunn et al. (199) observed no difference in NR figures between semen thawed at 40°C and semen thawed at 5°C. In contrast, a limited field trial (198) showed that fast thawing at 40°C and slow thawing at 5°C were both better than thawing at an intermediate temperature of 20°C. The differences were not statistically significant.

Although there is some disagreement on practical methods for thawing, it must be concluded from these data on rates of thawing for ampules containing 1 milliliter of semen, that thawing in water at 40°C or in ice water are the only methods that should be considered. The difficulty of keeping water at 40°C in the field, and the possibility of later cold shock if the ampules are held more than a few seconds at that temperature, eliminate 40°C. Thus thawing in ice water becomes the method of choice.

Current recommendations are to place at least a pint of water in an insulated container constructed so that ampules cannot freeze to the ice cubes or to each other (see Figure 17-18). If ampules freeze together or to ice cubes the rate of thawing could be altered. A typical thawing curve is presented in Figure 17-19. Although it is not known whether a critical temperature range analogous to that during freezing affects thawing survival, it is at this point that the rate of rewarming begins to change (see Figure 17-19). Thus thawing procedure could be critical.

Eight to 10 minutes should be allowed for the ampule to thaw and it should not be disturbed during that time. This recommendation differs from that of Boyd and Hafs (200). They compared motility and fertility of spermatozoa in ampules from which the "ice coat" was removed (fast thawing) to that of ampules allowed to thaw undisturbed in ice water (slow thawing). There were no statistically significant differences in motility or fertility between the methods. The results of two field trials, however, favored the slow thawing technique by 0.6 and 2.0 percentage points in NR, respectively.

From the results presented in Figure 17-20, it is recommended that the semen be used as soon as possible after thawing; if it is not used within one hour it should be discarded. This point is not universally agreed upon, however (201).

Thawing in Straws. The principles involved in thawing semen stored in straws should be similar to those that apply to semen stored in ampules. However,

the heat transfer is different than the technique used at 4°C, or in ice water and thawing in seconds.

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the heat transfer characteristics of a long, slender plastic container require different thawing techniques. Aamdal and Andersen (159) used a staining technique to evaluate the results of thawing semen in straws. Thawing in water at 4°C, or in water or air at 20°C, was inferior to thawing in water at 35°C; and thawing at 35°C for 30 seconds was inferior to thawing at 75°C for 12 seconds.

French investigators (141, 148, 189) who were responsible for many of the techniques employed with the straw, thaw them in water at 35°C. In contrast, Canadian workers (155) thaw the 0.5-milliliter straw by "rubbing them back

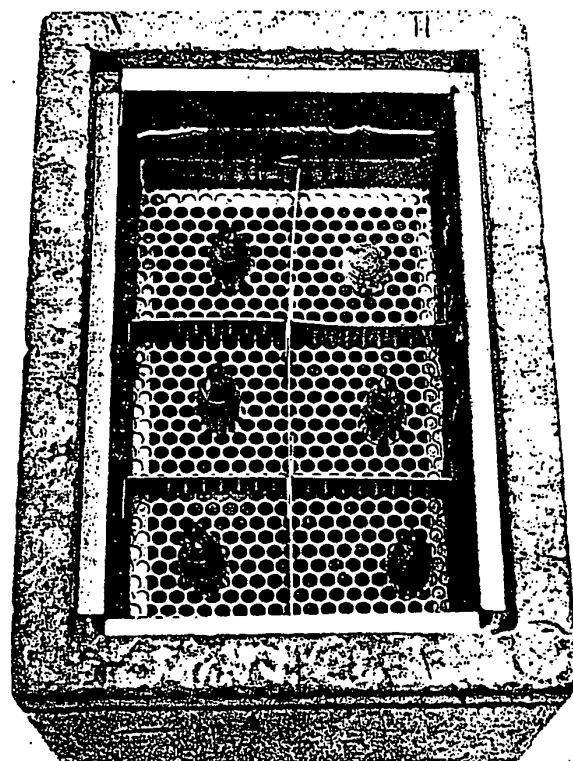


Figure 17-18. A thawing container designed to prevent ampules from freezing together or to ice cubes.

and forth between the palms of the hands." In a study by Bean (149) 16,000 to 21,000 first service inseminations per treatment were performed with semen in 0.5-milliliter straws thawed in ice water, warm water (30 to 35°C), or air. The 60-90-day NR rates were 71.1, 72.7, and 72.8 percent for thawing in ice water, warm water, and air, respectively. No statistical analyses were reported, but air thawing by wrapping the straw in a paper towel and placing it in the inside pocket of a technician's coveralls was adopted.

Robbins et al. (202) thawed semen in straws by plunging them into water baths maintained at temperatures of 5, 20, 35, or 75°C. In general, the more rapid thawing rates resulted in better motility and retention of the acrosomal cap (249). These results (202) disagree with the fertility results obtained by Bean (149). Although the ultimate test of a procedure is fertility (141), the results obtained by Robbins et al. (202) conform to the theoretical expectation—the faster semen is frozen the faster it should be thawed.

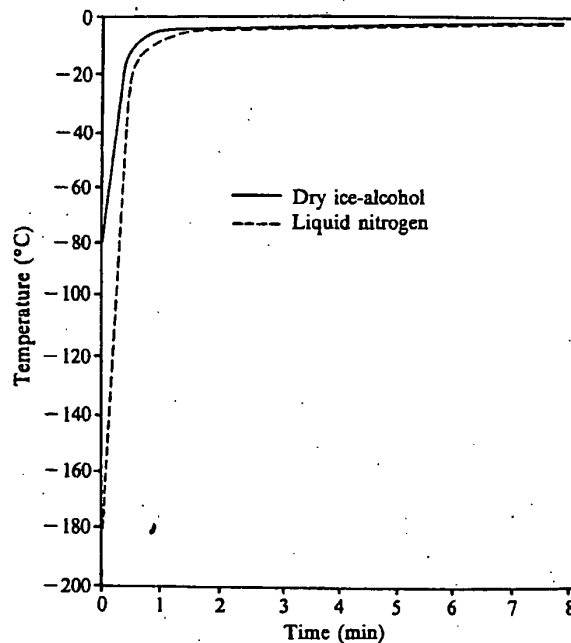


Figure 17-19. Typical thawing curve from -79° and -196°C for 1.0-milliliter glass ampules of bovine semen in ice water. Thawing rate measured by copper-constantan thermocouple located in the center of the ampule. [From Pickett. *A.I. Digest* 19(2):8-23. 1971.]

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17-6 VARIATION IN SEMEN QUALITY

The individual bulls producing the semen are a major source of variation in all frozen semen experiments. Among the factors that can contribute to this effect are genetics, age, health, frequency of collection, and degree of sexual preparation (213, 218). Dairy and beef bulls vary in the quality of semen they produce and in the processing techniques needed for optimum fertility (219). Within types the heritability estimates of fertility in bulls vary (203, 204). However, the genetic variance of male fertility is relatively small and the selection pressure necessary to improve inherent breeding efficiency would be very high and could be made at the expense of other economically important traits (205). The major emphasis in commercial AI should therefore probably be directed to methods of obtaining and maintaining semen of greatest possible quality from available animals.

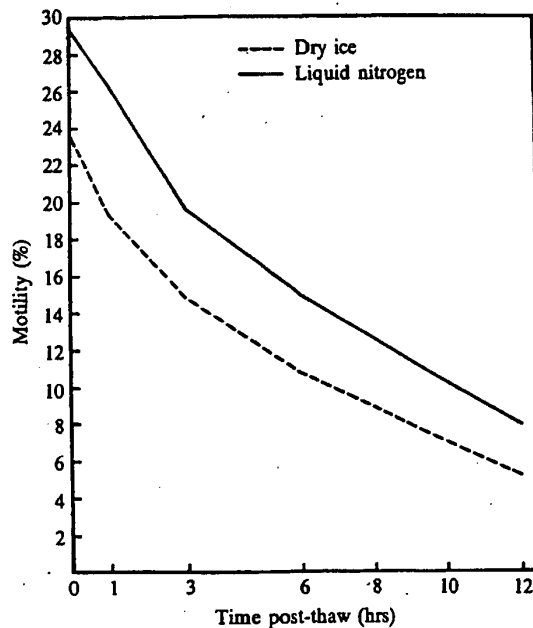


Figure 17-20. The effect of storage time at 5°C after thawing on percent motility of bovine spermatozoa. [From Pickett. *A.I. Digest* 19(2):8-23. 1971.]

The quality and fertility of semen produced declines as a bull ages (206, 207). Theoretically, semen can be collected from bulls at the height of their fertility and then frozen and stored for subsequent use. However, economic considerations and the delays inherent in evaluating and adopting innovations in semen processing and handling may preclude such policies (208).

The ability of semen to be frozen successfully, as indicated by post-thaw motility of several successive ejaculates, is often higher in the second ejaculate than in the first (210) and increases up to the fourth (209), especially if the semen has not been collected frequently on a regular schedule. For collection after 21 to 30 days of sexual rest the fertility of several consecutive ejaculates were not significantly different statistically, though the means favored the second (211). Short intervals between collection periods tend to eliminate the differences in freezability between two consecutive ejaculates (125). Proper sexual preparation has the same effect (212). The differences among ejaculates of the same bull are due to differences in the age distribution of individual sperm cells within ejaculates; this in turn affects the mean chemical composition (71, 72, 214, 215, 217) and resistance to cold shock (216). It is likely that some bulls are by-passed for AI because their semen freezes poorly under routine procedures that could be improved by generating special processing, freezing, and thawing procedures.

The number of cows to be bred each month of the year influences the amount of semen to be collected, processed, and frozen. The breeding of beef cows is confined to a relatively short season, usually in early spring or summer, depending on weather conditions and latitude. Dairy cows are bred more regularly throughout the year. Bulls proven to be genetically exceptional will undoubtedly be collected as frequently as possible, regardless of season. However, young bulls and bulls from which only a limited number of semen units are required should be collected during the time of the year when the semen is most fertile.

Salisbury (183) has presented evidence that at approximately 39° to 40° north latitude the observed seasonal variation in fertility is due primarily to an effect on the bulls of the seasonal differences in climate. The semen used for this study was frozen and stored at -79° to -88°C. The fertility of semen collected from May through October was significantly lower than that of semen collected during the other 6 months (see Table 17-10). These observations of decreased fertility in the summer months have been corroborated (191). In addition, Sullivan and Elliott (220) have found that semen collected in the fall and winter from bulls housed in a standard temperature-controlled environment and stored at -196°C was significantly higher in fertility (76.3 vs 74.2 percent, 60-90-day NR) than semen collected in the spring and summer (see Figure 17-21).

Custom freezing of semen is highly seasonal. Too often an owner will not present a bull for freezing of the semen until shortly before it is needed. This

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is not good. Several ejaculates often must be collected before semen is obtained that is suitable for freezing. Waiting until the semen is needed could thus result in costly delays in the breeding schedule or in having to use a bull of less genetic potential.

From the results of these studies (183, 191, 220) it is recommended, economics and genetics notwithstanding, that semen should be collected for freezing during the fall and winter.

Table 17-10. The decrease in fertility (mean difference in 167-day nonreturns) between 1 and 2 months' storage and > 6 months' storage at about -79°C for the months in the year of insemination (cow response) and of semen collection (bull response).^a

Month	Cow response	Bull response
	Decrease after > 6 mo. storage	Decrease after > 6 mo. storage
	(%)	(%)
January	13.8	7.0
February	13.8	11.8
March	16.6	8.4
April	13.5	11.5
May	12.8	16.8 ^b
June	13.2	16.7 ^b
July	11.3	20.9 ^b
August	14.4	14.2 ^b
September	7.3	21.4 ^b
October	13.1	15.7 ^b
November	14.6	12.8
December	14.7	8.9
Total insemin.	174,307	176,432
Mean decrease	13.3	13.3

^aFrom Salisbury. 6th Inter. Congr. Anim. Reprod. and Artif. Insem. Paris. II:1189-1204. 1968 (183).

^b $P = 0.01$ that difference from mean is not due to chance.

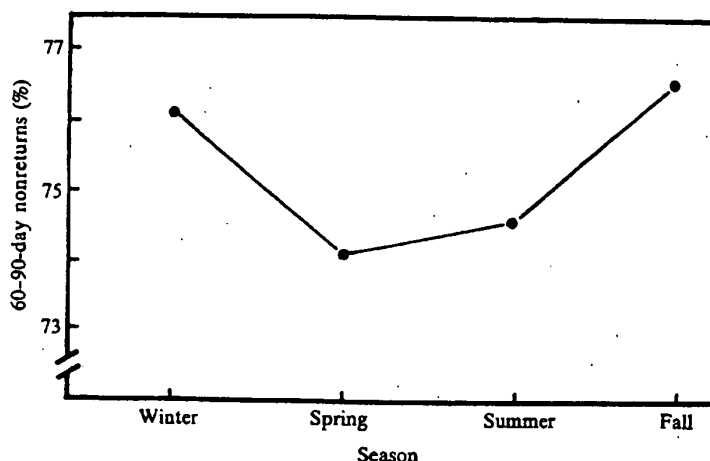


Figure 17-21. The effect of season on 60- to 90-day % NR [From Sullivan and Elliott. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. I:329-332. 1968.]

17-7 FREEZE-DRYING

The long term storage and subsequent shipment of spermatozoa at -196°C is costly and often inconvenient. If semen could be dried, placed in small packages, and stored at room temperature and still retain its fertility monumental savings could be realized. Attempts to recover motile spermatozoa following freeze-drying have met with variable success (9, 221-227, 238, 250). Meryman and Kafig (221) performed a single successful insemination with freeze-dried bull sperm, but they were unable to repeat this success (222). More recently, Graham et al. (250) have reported another successful insemination with freeze-dried sperm. It is believed that earlier attempts to freeze-dry semen failed because of the presence of glycerol in the extending medium. It is also true that the studies reporting successful recovery of motility upon reconstitution have been under conditions in which the semen samples have not been taken to a steady state of dryness (0.5 to 2.5 percent moisture) but have a higher moisture content. Very little success has been reported after the freeze-dried semen has been stored for a while; this may be related to the fact that when sperm cells are taken to a steady state of dryness motility cannot be recovered upon reconstitution with water. Reliable procedures for preserving bull spermatozoa by freeze-drying are not currently available, and much time and research are still necessary before the procedure will be of practical use.

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The basic principles and techniques set forth in this chapter are intended to provide guidelines for obtaining maximum reproductive efficiency in a frozen semen program. Recommendations have been made according to available research data. While these techniques should provide optimal results under most circumstances, an awareness of the special needs of spermatozoa from individual bulls, and the flexibility required to meet these needs, should accompany every AI program.

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18-1 INTRODUC

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICATION NO:	09/478,299
	FILINGDATE:	1/5/2000
	FIRSTNAMEDINVENTO	John L. Schenk
	ARTUNIT:	1654
	EXAMINERNAME:	M. Meller
	DOCKETNO:	22091-701

I. US PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO. & KIND CODE (if known)	PATENTEE OR APPLICANT NAME	PUBLICATION/ISSUE DATE	Pages, Columns, Lines Where Relevant Passages Or Relevant Drawings Appear
	2005/0003472 A1	Muhammad, A.	1/6/2005	
	2005/0112541 A1	Durack, G.	5/26/2005	
	2005/0214733 A1	Graham, J.A.	9/29/2005	

II. FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL	Foreign Patent Document Country Code, Number, Kind Code (if known)	PATENTEE OR APPLICANT NAME	PUB'NDATE dd-yyyy	TRANSLATION	
				Yes	No
	WO 04/017041 A3 Search Report	XY INC.	2/26/2004		
	WO 04/012837 A3 Search Report	XY, Inc.	2/12/2004		
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III. OTHER REFERENCES

EXAMINER INITIAL	Document
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Examiner	Date Considered
EXAMINER: Please initial if citation considered, whether or not citation is in conformance with MPEP §	

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IN THE UNITED STATES PATENT AND
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Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

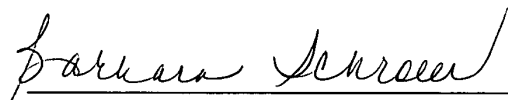
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<u>L6</u>	extender	66464	<u>L6</u>
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=> s cryopreserv?

L2 32071 CRYOPRESERV?

=> s sex

L3 703536 SEX

=> s extender

L4 9579 EXTENDER

=> s l1 and l2 and l3 and l4

L5 0 L1 AND L2 AND L3 AND L4

=> s sperm

L6 147014 SPERM

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L7 2 L2 AND L3 AND L4 AND L6

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AB Expts. were performed to validate an extraction technique for canine acrosin (EC 3.4.21.10) and to quantitate the acrosin activity of fresh and frozen-thawed spermatozoa. Acrosin activity from fresh spermatozoa differed amongst dogs and was influenced by the interval since previous ejaculation. Freezing and thawing spermatozoa induced a loss of acrosin activity that differed with the **extender** in which the spermatozoa were frozen. The assay of acrosin activity, in conjunction with motility ests., provides a more complete evaluation of the efficacy of seminal extenders in attenuating freezing injury than do motility ests. alone.

AN 100:83444 CA

TI Acrosin activity of canine spermatozoa as an index of cellular damage

AU Froman, D. P.; Amann, R. P.; Riek, P. M.; Olar, T. T.

CS Anim. Reprod. Lab., Colorado State Univ., Fort Collins, CO, 80523, USA

SO Journal of Reproduction and Fertility (1984), 70(1), 301-8

CODEN: JRPFA4; ISSN: 0022-4251

DT Journal

LA English

L7 ANSWER 2 OF 2 BIOSIS COPYRIGHT (c) 2006 The Thomson Corporation on STN

AB Black sea bass *Centropristis striata* L. are protogynous hermaphrodites that develop and spawn as females before changing **sex** to male. Since all fish eventually become males, determining the relationship between **sperm** production, **sperm** quality and seasonal changes in plasma levels of testosterone (T) and 11-ketotestosterone (11-KT) could be useful for identifying appropriate males to maintain as broodstock. Milt and blood samples were collected three times during an 8-week spawning season. Milt volume (3.5+/-0.76 mL kg⁻¹), **sperm** density (3.2 x 10⁸+/- 0.31 cells mL⁻¹), **sperm** production (11 x 10⁸+/-3.4 cells kg⁻¹ body weight (BW)) and **sperm** motility (80+/-0.6%) were at their highest during the first sampling interval and coincided with the highest 11-KT levels (1.0+/- 0.11 ng mL⁻¹). All of the **sperm** indices decreased to their lowest levels during the final 3 weeks of the study. **Sperm** viability was highly correlated (adjusted R²=0.84) with **sperm** motility. **Sperm cryopreserved** in modified Mounib's **extender** (MME) had the highest post-thaw motility compared with two other extenders. Post-thaw motility of **sperm cryopreserved** in MME was not different from fresh after 90 days of storage. There was no

difference in fertilization rates between fresh (69+/-2.4%) and post-thaw (67+/-4.1%) **sperm** samples taken from the same male or among males. These results demonstrate that the quality of black sea bass spermatozoa is higher earlier in the spawning season and that acceptable post-thaw fertilization rates can be obtained from **cryopreserved sperm**.

AN 2005:102134 BIOSIS
DN PREV200500099168
TI Production and storage of **sperm** from the black sea bass
Centropristis striata L.
AU DeGraaf, James Dana; King, William; Benton, Christopher; Berlinsky, David
L. [Reprint Author]
CS Dept Zool, Univ New Hampshire, Durham, NH, 03824, USA
david.berlinsky@unh.edu
SO Aquaculture Research, (December 22 2004) Vol. 35, No. 15, pp. 1457-1465.
print.
ISSN: 1355-557X (ISSN print).
DT Article
LA English
ED Entered STN: 9 Mar 2005
Last Updated on STN: 9 Mar 2005



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509
	7590	02/09/2006	EXAMINER	
Craig Miles Santangelo Law Offices P C 125 South Howes Street 3rd Floor Fort Collins, CO 80521			MELLER, MICHAEL V	
			ART UNIT	PAPER NUMBER
			1655	

DATE MAILED: 02/09/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No. 09/478,299	Applicant(s) SCHENK, JOHN L.	
	Examiner Michael V. Meller	Art Unit 1655	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --
Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
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- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 14 November 2005 and 20 January 2006.
2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.
3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 38-65 and 67 is/are pending in the application.
4a) Of the above claim(s) 40,41,55,56,60-62,65 and 67 is/are withdrawn from consideration.
5) ☐ Claim(s) _____ is/are allowed.
6) ☒ Claim(s) 38,39,42-54,57-59,63 and 64 is/are rejected.
7) ☐ Claim(s) _____ is/are objected to.
8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)	4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)	5) ☐ Notice of Informal Patent Application (PTO-152)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____	6) ☐ Other: _____

DETAILED ACTION

Election/Restrictions

The election/restriction of record is maintained for the reasons of record.

Claims 40, 41, 55, 56, 60-62, 65, 67 remain withdrawn from further consideration as being drawn to non-elected subject matter.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claim Rejections - 35 USC § 112

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

There is still no support in the specification for the ranges of sperm cells claimed. There is no support for limitations such as, "to at least about 5 million per milliliter to at least about 10 million per milliliter of extender", "equine sperm cells between about 1,000,000 and about 25,000,000".

The specification may mention specific points of the concentration of sperm cells (i.e. 5×10^6) but it does not provide for the claimed range of the concentrations (i.e. between about 1,000,000 and about 25,000,000). The concentrations in table 1 are noted but they are only isolated concentrations, there is no support for ranges of these concentrations, only the specific points tested. The specification is also noted by applicant on page 11, lines 25-28 as teaching concentration ranges from 1 million-300 million, 10 million to 50 million, and 10 million to 20 million, but not the ranges applicant has claimed. Thus, the claims need to be amended to the ranges for which they find support for in the instant specification.

The time limitation in claim 64 still has no support. In fact, as applicant noted, they have support for 1-18 hours and between about 3 and about 6 hours but not support for "not greater than 6 hours".

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Further, what is meant by , "to at least about 5 million per milliliter to at least about 10 million per milliliter of extender" ? Is this the concentration of the extender before it is applied to the sperm cells ? Is the 5 million referring to the number of sperm cells. So how much extender is added to each sperm cell ?

This was never addressed by applicant.

Cooling is still not clear in claim 38. Cooling to what temperature ? Cooler than what ?

Claims 63 and 64 are confusing. What step is this referring to ? What steps does this step occur between ? It might be clearer if applicant refers to the steps by the letters they correlate from in claim 38, i.e. step b. What temperature is cooler ? Cooler than what ? This is relative and subjective. There is no point of reference for one to compare it to.

Claim Rejections - 35 USC § 103

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. in view of Spaulding and Shrimpton.

The claims still are obvious over the cited prior art of record. Applicant's limitation in claim 38 of "sorting said sperm cells, without the presence of protective compounds in seminal plasma" is met since as applicant notes on page 10 of their response filed 2/28/2005, that flow cytometry which is used by Spaulding has been shown to work and is now in practice as applicant has also done. Applicant states on page 10 that sorting

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without the presence of protective compounds is a feature that occurs when flow cytometry is used to achieve sex sorting which Spaulding has also done just like applicant and this is of record. Thus, the references meet the claim limitations.

Applicant also argues that the concentrations of the sperm cells in the extenders is 5 million to 10 million and applicant has pointed to table 1 of the specification to support this, but these points go up and down. For example, the values go up as one approaches 10 million and then decreases as one goes above 10 million. Also, it is not clear what this signifies. It is not clear what significance the increase in the numbers means. Does this increase anything ? Just because one yields higher numbers at 10 million this does not correlate to anything and if it does what does it correlate to ? Further, it is not clear what the 61 refers to in relation to 10 million in table 1. Also it is noted that the values at 24 hours and 48 hours differ in some way. It is not clear what this signifies.

Applicant has submitted a declaration by John Schenk filed 1/20/2006. While Mr. Schneck's declaration is noted what is not clear is how he can summarily dismiss the findings and teachings in a US Patent. Mr. Schenck argues that he has never observed a successful accomplishment of the techniques described in the Spaulding reference. While this may be true, it does not take away from the findings and teachings of Spaulding. Flow cytometry was well known in the art at the time this invention was made to sort sperm cells based on the DNA content of the sperm cell. For Mr. Schneck to say that he has seen no accomplishment of the Spaulding reference is only his opinion and does not mean that Spaulding is inoperable as a reference. Further, the

declaration argues that the concentrations of the sperm in the extender being from about "at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender" is not well taken since applicant does not have the data in his own specification to back that allegation up. As noted above, applicant only has support in the specification for either 10 million to 20 million sperm per milliliter or isolated points in table 1, 1.25 million sperm, 2.5 million sperm, 5 million sperm, 10 million sperm, 15 million sperm, and 20 million sperm. Nowhere in the specification or the original claims does applicant have support for the claimed "at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender". Without such evidence of support for the allegations in the declaration can it be given any weight or merit.

Applicant has further argued that in Spaulding the sperm are stripped of their natural protective compounds and are thus, as applicant concludes, subjected to a more stressful environment. Applicant states on page 10 of their response filed 2/2/8/2005 that sorting without the presence of protective compounds is a feature that occurs when flow cytometry is used to achieve sex sorting which Spaulding has also done just like applicant and this is of record. Thus, this argument is simply moot.

This is a RCE of applicant's earlier Application No. 09/478,299. All claims are drawn to the same invention claimed in the earlier application and could have been finally rejected on the grounds and art of record in the next Office action if they had

been entered in the earlier application. Accordingly, **THIS ACTION IS MADE FINAL** even though it is a first action in this case. See MPEP § 706.07(b). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire **THREE MONTHS** from the mailing date of this action. In the event a first reply is filed within **TWO MONTHS** of the mailing date of this final action and the advisory action is not mailed until after the end of the **THREE-MONTH** shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no, however, event will the statutory period for reply expire later than **SIX MONTHS** from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 571-272-0967. The examiner can normally be reached on Monday thru Thursday: 9:30am-6:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Terry McKelvey can be reached on 571-272-0775. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

A handwritten signature in black ink, appearing to read 'M. Meller', followed by a horizontal line extending to the right.

Michael V. Meller
Primary Examiner
Art Unit 1655

MVM



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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICATION NO:	09/478,299
	FILING DATE:	1/5/2000
	FIRST NAMED INVENTOR:	John L. Schenk
	ART UNIT:	~~3854~~ 1655
	EXAMINER NAME:	M. Meller
	DOCKET NO:	22091-701

I. US PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO. & KIND CODE (if known)	PATENTEE OR APPLICANT NAME	PUBLICATION/ISSUE DATE	Pages, Columns, Lines Where Relevant Passages Or Relevant Drawings Appear
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II. FOREIGN PATENT DOCUMENTS

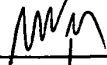
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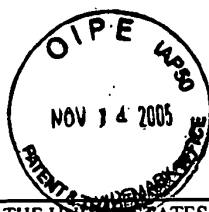


III. OTHER REFERENCES

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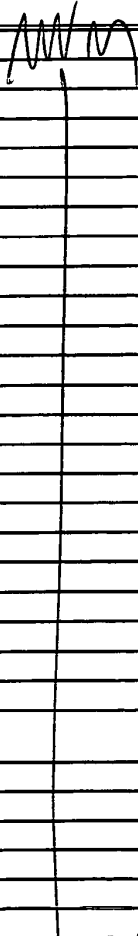
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Examine	Date Considered
EXAMINER: Please indicate if citation considered, whether or not citation is in conformance with MPBP 8	

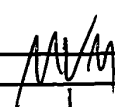


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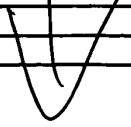
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICATIONNO:	09/478,299
	FILINGDATE:	1/5/2000
	FIRSTNAMEDINVENTOR	John L. Schenk
	ARTUNIT:	~~4064~~ 1655.
	EXAMINERNAME:	M. Meller
	DOCKETNO:	22091-701

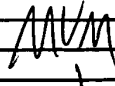
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EXAMINER INITIAL	DOCUMENT NO. & KIND CODE (if known)	PATENTEE OR APPLICANT	PUBLICATION/ISSUE DATE	Pages, Columns, Lines Where Relevant Passages Or Relevant Drawings Appear
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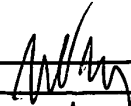




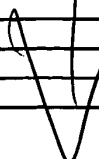
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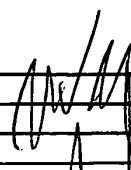


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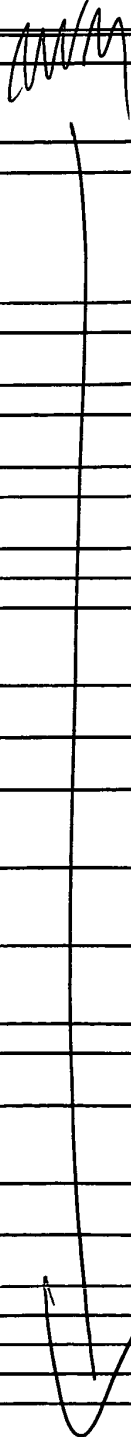




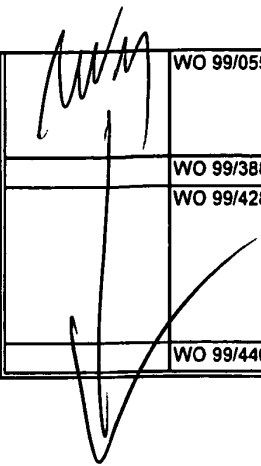
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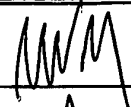
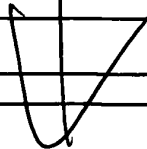
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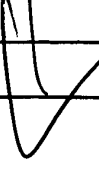


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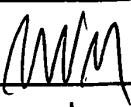
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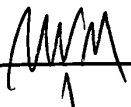
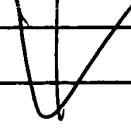
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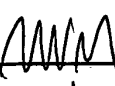
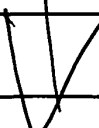
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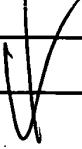
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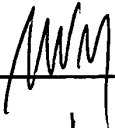
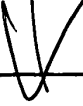
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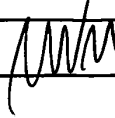
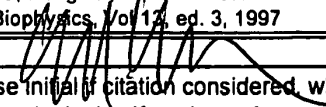
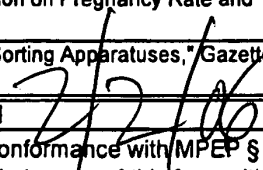
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Examiner 	Date Considered 
EXAMINER: Please initial if citation considered, whether or not citation is in conformance with MPEP § 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.	

Search Notes

Application/Control No.

09/478,299

Examiner

Michael V. Meller

Applicant(s)/Patent under
Reexamination

SCHENK, JOHN L.

Art Unit

1655

SEARCHED

Class	Subclass	Date	Examiner
Updated 2/2/06 mm			

INTERFERENCE SEARCHED

Class	Subclass	Date	Examiner

**SEARCH NOTES
(INCLUDING SEARCH STRATEGY)**

	DATE	EXMR
Updated 2/2/06 mm		

Index of Claims



Application/Control No.

09/478,299

Examiner

Michael V. Meller

Applicant(s)/Patent under Reexamination

SCHENK, JOHN L.

Art Unit

1655

✓	Rejected
=	Allowed

-	(Through numeral) Cancelled
+	Restricted

N	Non-Elected
I	Interference

A	Appeal
O	Objected

Claim	Date
Final	Original
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2	2
3	2
4	2
5	2
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PATENT APPLICATION FEE DETERMINATION RECORD
Effective October 1, 2003

Application or Docket Number:

09/478299

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)
TOTAL CLAIMS		
FOR	NUMBER FILED	NUMBER EXTRA
TOTAL CHARGEABLE CLAIMS	13 minus 20 =	-
INDEPENDENT CLAIMS	1 minus 3 =	-
MULTIPLE DEPENDENT CLAIM PRESENT ☐		

* If the difference in column 1 is less than zero, enter "0" in column 2

CLAIMS AS AMENDED - PART II

9-7-05

	(Column 1)	(Column 2)	(Column 3)
AMENDMENT A	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	13	Minus 20	=
Independent	1	Minus 3	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐			

SMALL ENTITY TYPE ☐

OR OTHER THAN SMALL ENTITY

RATE	FEE	RATE	FEE
BASIC FEE	340	BASIC FEE	
XS 9=		XS 18=	
X43=		X86=	
+145=		+290=	
TOTAL	340	TOTAL	

SMALL ENTITY

OR OTHER THAN SMALL ENTITY

RATE	ADDI-TIONAL FEE	RATE	ADDI-TIONAL FEE
XS 9=		XS 18=	
X43=		X86=	
+145=		+290=	
TOTAL ADDIT. FEE		TOTAL ADDIT. FEE	

5/24/06

	(Column 1)	(Column 2)	(Column 3)
AMENDMENT B	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	9	Minus 20	=
Independent	1	Minus 3	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐			

RATE	ADDI-TIONAL FEE	RATE	ADDI-TIONAL FEE
XS 9=		XS 18=	
X43=		X86=	
+145=		+290=	
TOTAL ADDIT. FEE		TOTAL ADDIT. FEE	

	(Column 1)	(Column 2)	(Column 3)
AMENDMENT C	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total		Minus	=
Independent		Minus	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐			

RATE	ADDI-TIONAL FEE	RATE	ADDI-TIONAL FEE
XS 9=		XS 18=	
X43=		X86=	
+145=		+290=	
TOTAL ADDIT. FEE		TOTAL ADDIT. FEE	

- * If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
- * If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
- * If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."
- The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.



Express Mail No: EV 673965651 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1655
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

**REQUEST FOR CONTINUED EXAMINATION
PURSUANT TO 37 C.F.R. § 1.114**

I. INTRODUCTORY REMARKS

The Assignee hereby submits the following submission in this case as a Request for Continued Examination pursuant to 37 C.F.R. § 1.114. This submission consists of a request for reconsideration and a response to the Office Action dated February 9, 2006. This Request for Continued Examination is appropriate because:

- i) this request is made prior to payment of the issue fee [37 C.F.R. § 1.114 (a)(1)];
- ii) the application has not been abandoned [37 C.F.R. § 1.114 (a)(2)];
- iii) no notice of appeal has been filed [37 C.F.R. § 1.114 (a)(3)];
- iv) prosecution on the application is closed by either a final action or issuance of a notice of allowance [37 C.F.R. § 1.114 (b)];
- v) this application is not a provisional application [37 C.F.R. § 1.114 (e)(1)];
- vi) this is not a utility patent application filed before June 8, 1995 [37 C.F.R. § 1.114 (e)(2)];
- vii) this application is not an international application filed under 35 U.S.C. § 363 before June 8, 1995 [37 C.F.R. § 1.114 (e)(3)];

- viii) this application is not a design patent application [37 C.F.R. § 1.114 (e)(4)]; and
- ix) this is not a patent under reexamination [37 C.F.R. § 1.114 (e)(5)].

Amendments to the Claims begin on page 3 of this paper.

Remarks begin on page 10 of this paper.

A shortened statutory period of three months has been set, making a response to this action due on or by May 9, 2006. Pursuant to 37 C.F.R. §1.136(a), a petition for extension of time is submitted herewith extending the period for three months to and including August 9, 2006 along with the appropriate fee. The Assignee submits this request for reconsideration to fully address the Office's February 09, 2006 action.

II. AMENDMENTS TO THE CLAIMS:

This listing of claims will replace all prior versions, and listings, of claims in the application.

Fees in the amount of \$100.00 are included for the additional claims as follows:

Added claims: 2 @ \$50.00 = \$100.00

Added Independent Claims: 2

Total Independent Claims: 3

Independent claims in excess of 3: 0

Total Fee paid:	\$100.00

Listing of Claims:

Claims 1-37 (Cancelled)

38. (Currently Amended)

A method of cryopreserving sex-selected sperm cells, comprising:

- a. obtaining sperm cells from a species of a non-human male mammal;
- b. sorting said sperm cells, without the presence of protective compounds in seminal plasma, and based upon sex-type to create a collection of sex-selected sperm cells using flow cytometry or fluorescence-activated cell sorting;
- c. cooling said sex-selected sperm cells;
- d. suspending said sex-selected sperm cells in an extender to ~~at least~~ about 5 million per milliliter of extender to ~~at least~~ about 10 million per milliliter of ~~extender~~, and; extender; and
- e. freezing said sex-selected sperm cells in said extender.

39. (Currently Amended)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said non-human male mammal are selected from the

group consisting of bovine sperm cells and equine sperm cells.

40. (Withdrawn)

A method of cryopreserving sperm cells as described in claim 39, and further comprising the step of isolating a number of bovine sperm cells between about 300,000 and about 3,000,000.

41. (Withdrawn)

A method of cryopreserving sperm cells as described in claim 39, and further comprising the step of isolating a number of bovine sperm cells of no more than about 1,000,000.

42. (Currently Amended)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said non-human male mammal comprise equine sperm cells.

43. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 42, and further comprising the step of isolating a number of equine sperm cells between about 1,000,000 and about 25,000,000.

44. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 42, and further comprising the step of isolating a number of equine sperm cells of no more than about 5,000,000.

45. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said step of cooling sex-selected sperm cells comprises reducing the temperature of said sex- selected sperm cells to about 5°Celsius.

46. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 45, wherein said step of reducing the temperature of said sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells for a period of about 60 minutes to about 240 minutes.

47. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said extender further comprises a component which maintains osmolality and buffers pH.

48. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.

49. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris[hydroxymethyl]aminomethane, 200mM Tris[hydroxymethyl]aminomethane, 175 mM to 225mM Tris[hydroxymethyl]aminomethane, 200 mM Tris[hydroxymethyl]aminomethane/65mM citric acid monohydrate, 175 mM to 225mM Tris[hydroxymethyl]aminomethane/50mM to 70mM citric acid monohydrate, N-Tris [hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/65 mM citric acid monohydrate, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/50mM to 70 mM citric acid monohydrate, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

50. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 47, 48, or 49, wherein said extender has a pH in the range of about 6.5 to about 7.5.
51. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 50, wherein said extender further comprises a cold shock protectant.
52. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 51, wherein said cold shock protectant is selected from the group consisting of egg yolk, 20% egg yolk, 15% to 25% egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.
53. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 51, wherein said extender further comprises an energy source.
54. (Previously Presented)
A method of cryopreserving sex-selected sperm cells as described in claim 53, wherein said energy source is selected from the group consisting of a saccharide, glucose, fructose, 56 mM fructose, 45mM to 60mM fructose, mannose, and any combination thereof.
55. (Withdrawn)
A method of cryopreserving sex-selected sperm cells as described in claim 53, wherein said extender further comprises an antibiotic.
56. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 55, wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.

57. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, 51, 53, or 55, wherein said extender further comprises a cryoprotectant.

58. (Currently Amended)

A method of cryopreserving sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any ~~combination~~ combination thereof.

59. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of glycerol, 6% glycerol, between 5% to 7% glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.

60. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

61. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, egg yolk, and one or more antibiotics.

62. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, egg yolk, milk, fructose, and one or more antibiotics.

63. (Currently Amended)

A method of cryopreserving sex-selected sperm cells as described in claim 38, further comprising a step of equilibrating said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to said freezing step (e) for a period of about 1 hour to about 18 hours.

64. (Currently Amended)

A method of cryopreserving sex-selected sperm cells as described in claim 59, further comprising a step of equilibrating said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to said freezing step (e) over a period of not greater than 6 hours.

65. (Withdrawn)

A frozen sex-selected sperm sample in accordance with the method of claim 38.

66. (Cancelled)

67. (Withdrawn)

A method of cryopreserving sperm cells as described in claim 38, wherein said step of freezing said sex-selected sperm cells in said extender comprises freezing a number of bovine sperm cells between about 300,000 and about 5,000,000.

68. (New)

A method of cryopreserving sex-selected sperm cells, comprising:

- a. obtaining sperm cells from a species of a non-human male mammal;

- b. sorting said sperm cells, without the presence of protective compounds in seminal plasma, and based upon sex-type to create a collection of sex-selected sperm cells;
- c. cooling said sex-selected sperm cells;
- d. suspending said sex-selected sperm cells in an extender to at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender; and
- e. freezing said sex-selected sperm cells in said extender.

69. (New)

A method of cryopreserving sex-selected sperm cells, comprising:

- a. obtaining sperm cells from a species of a non-human male mammal;
- b. sorting said sperm cells, without the presence of protective compounds in seminal plasma, and based upon sex-type to create a collection of sex-selected sperm cells;
- c. cooling said sex-selected sperm cells;
- d. suspending said sex-selected sperm cells in an extender to about 5 million per milliliter of extender to about 20 million per milliliter of extender; and
- e. freezing said sex-selected sperm cells in said extender.

III. REMARKS

The amendments submitted herein should be understood to be made as a practicality only, and should not to be construed as creating any situation of file wrapper estoppel or the like as all rights are expressly reserved and may be pursued in this or other applications, such as divisionals, continuations, or continuations-in-part if desired. Relatedly, it should be understood that the amendments made herein are made for tangential issues of clarity and as a matter of the Office's convenience or expedience only. The amendments should not be interpreted as an action that in any way surrenders a particular equivalency, surrenders any right to patent coverage, or otherwise limits any rights which the Assignee may now or hereafter assert. It should be understood that, unless and to the extent deemed broadened by this amendment, and even as amended, the Assignee expressly reserves all rights, including but not limited to: all rights to maintain the scope of literal coverage with respect to any element as may have existed under the language previously presented, all rights to maintain the scope of equivalency coverage as may have existed under the language previously presented, and all rights to re-present the prior language at any time in this or any subsequent application. To the extent currently foreseeable, no change or reduction in direct or equivalency coverage is believed to exist, and no change or reduction in direct or equivalency coverage is intended through the presentation of this amendment.

The Assignee amends the claims to convey that that the sperms cells are from a species of a non-human male mammal.

35 U.S.C. § 112 Concerns

The action expressed concerns to the claims under 35 U.S.C. § 112 First and Second Paragraphs. Claim 38 and newly added claims 68 and 69 reference the aspect of sperm cells in an extender suspended to at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender, about 5 million per milliliter of extender to about 10 million per milliliter of extender, or about 5 million per milliliter of extender to about 20 million per milliliter of extender. The values and results shown in Table 1 on page 21 and to some degree in

Table 2 on page 19 highlight how the claimed ranges show significance in the data. Referencing Table 1, it can be seen how the results increase to a more consistent value when the dilution is established at values of at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender or higher. Thus, the Table 1 values support the claimed ranges as one skilled in the art can appreciate. Supplemental data will be provided to further support this.

In addition, to clarify the examiner's concerns regarding the reference to 5 million per milliliter and 10 million per milliliter of extender, Assignee specifies that these numbers relate to the concentration of sperm in an extender as discussed in the specification.

Claim 38 also references the aspect of cooling sex-selected sperm cells. As previously stated and to clarify the examiner's concerns regarding cooling, sperm cells can be cooled under suitable conditions within the level of skill in the art. As a non-limiting example, sperm can be cooled to 5 degrees Celsius. Of course, other temperatures may be used as one skilled in the art can appreciate.

Claim 43 references the aspect of utilizing between about 1,000,000 and about 25,000,000 equine sperm. As previously stated, various dosages and amounts are discussed in the specification. The volume of the straws used for insemination is also mentioned as both 0.25 ml and 0.50 ml at page 17 beginning on line 30 among other locations. Applying the extension mentioned in the specification at page 11 beginning at line 26 and at page 22 beginning at line 8 with these volumes of straws, it can be understood that sperm dosages of 1,250,000 (2.5×10^6 /ml in a 0.50 ml straw) and 25,000,000 (50×10^6 /ml in a 0.50 ml straw) were used. Further, at page 47 beginning at line 17, at page 48 beginning at line 5, at page 51 beginning at line 17, at page 52 beginning at line 25, and also at page 53 beginning at line 4, the use of 1,000,000 (1×10^6) sperm is explicitly mentioned. Assignee also notes that in the affidavit by John L. Schenk filed January 20, 2006, he stated that, "since the Original Application mentions concentrations of sperm of 1,000,000 and 25,000,000 (50×10^6 /ml in a 0.50 ml straw), the Original Application supports the claimed range of the concentrations of between about 1,000,000 to about 25,000,000 based on the substantial variation of doses that may be required." Accordingly,

support for the claimed ranges is found in the specification as one skilled in the art can appreciate.

Claim 64 mentions the aspect of equilibrating for a period of not greater than 6 hours. This aspect is found in the specification on page 17 lines 7-8. Assignee also notes that in the affidavit by John L. Schenk filed January 20, 2006, he stated that, “with respect to claim 64, I understand the text of the Original Application when it states, “to about 6 hours” to describe equilibrating for a period of not greater than 6 hours.” Accordingly, these claimed time limits are supported by the specification as one skilled in the art can appreciate. Further, the action expresses concerns to claims 63 and 64. Assignee believes that these concerns are clarified since both claims 63 and 64 now include, *inter alia*, “equilibrating ... *prior to said freezing step (e)* ...” (emphasis added).

In addition, claims 63 and 64 mention equilibrating sex-selected sperm cells to a cooler, non-freezing temperature. As previously stated and to clarify the examiner’s concerns regarding cooling, it may be desirable to cool the temperature of the sperm cells suspended in an extender to any temperature above freezing. As a non-limiting example, sperm can be cooled to 5 degrees Celsius. Of course, other temperatures may be used as one skilled in the art can appreciate.

35 U.S.C. § 103 Concerns

The action expressed concerns to the claims under 35 U.S.C. § 103. A prima facie case of obviousness requires that the combination of references disclose all of the limitations of the claimed invention. As the claims are currently stated, the prior art does not include all of the elements nor limitations of each of the independent claims 38, 68, or 69.

To respond to the action’s questions, Table 1 of the specification shows the sperm motility relationships for incubation times of 24 hours and 48 hours at different dilutions. Of course, sperm motility can decrease at 48 hours as compared to 24 hours, as one skilled in the art would appreciate. As previously stated, percentages of sperm motility increased as the sperm

concentration increased to 10×10^6 / ml (e.g. 61% at 24 hours), (more consistently between 5×10^6 / ml and 10×10^6 / ml) but there was little difference thereafter as seen in Table 1.

As previously noted, the claims are at this stage are distinguished from the references through their inclusion of the element of sorting the sperm cells without the presence of protective compounds in seminal plasma and suspending at dilutions of about 5 million per milliliter of extender to about 10 million per milliliter of extender, at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender, or about 5 million per milliliter of extender to 20 million per milliliter of extender. These techniques are not mentioned in the references at the dilutions as set forth. Assignee refers to the discussion above relating to the concentrations of sperm in the extender. Assignee also notes that in the affidavit by John L. Schenk filed January 20, 2006, it is noted the he "believe[s] that the techniques taught in the Spaulding and Shrimpton references are not capable of being successfully reproduced." Accordingly, the references do not address the aspects claimed and the section 103 concerns are not believed appropriate.

The Assignee believes all concerns raised in the February 9, 2006 office action have been addressed as best understood. Reconsideration and allowance of these remaining claims is respectfully requested at the Examiner's earliest convenience. Finally, should the Examiner have any remaining questions or disagree with any of Assignee's explanations, it is requested that the Examiner contact the undersigned by telephone in order to expedite the processing of this application.

Dated this 9 day of August, 2006.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.



Nicole A. Ressue
Attorney for Assignee
USPTO Reg. No. 48,665
125 South Howes, Third Floor
Fort Collins, CO 80521
(970) 224-3100

Express Mail No: EV 673965651 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

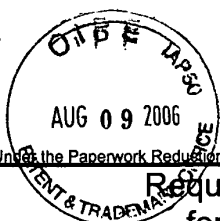
1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day deposited the attached Request for Continued Examination under 37 C.F.R. § 1.114 with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 9 day of August, 2006.



Cheryl A. Swanson



8-11-06

unpaid \$

PTO/SB/30 (07-06)

Approved for use through 09/30/2006. OMB 0651-0031

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

**Request
for
Continued Examination (RCE)
Transmittal**

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	09/478,299
Filing Date	01/05/2000
First Named Inventor	John L. Schenk
Art Unit	1654
Examiner Name	Michael V. Meller
Attorney Docket Number	22091-701

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. **Submission required under 37 CFR 1.114** Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

- a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.
- i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ii. ☐ Other _____
- b. ☒ Enclosed
- i. ☒ Amendment/Reply
- ii. ☐ Affidavit(s)/ Declaration(s)
- iii. ☒ Information Disclosure Statement (IDS)
- iv. ☐ Other _____

2. **Miscellaneous**

- a. ☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
- b. ☐ Other _____

3. **Fees**

The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

- The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. _____. I have enclosed a duplicate copy of this sheet.
- a. ☐
- i. ☒ RCE fee required under 37 CFR 1.17(e)
- ii. ☒ Extension of time fee (37 CFR 1.136 and 1.17)
- iii. ☐ Other _____
- b. ☐ Check in the amount of \$ _____ enclosed
- c. ☒ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	<i>Nicole A. Ressue</i>	Date	<i>August 9, 2006</i>
Name (Print/Type)	Nicole A. Ressue	Registration No.	48,665

CERTIFICATE OF MAILING OR TRANSMISSION

Express

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as ~~first class~~ mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Signature	<i>Cheryl A. Swanson</i>	Date	<i>8/9/2006</i>
Name (Print/Type)	Cheryl A. Swanson		

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

08/14/2006 GWORDF1 00000079 09478299

01 FC:1801

790.00 DP

02 FC:1202

100.00 DP



Express Mail No: EV 673965651 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.
2. I have this day deposited the attached a Request for Continued Examination (RCE) Transmittal with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 9 day of August, 2006.



Cheryl A. Swanson



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Application Number: 09/478,299
Applicants: John L. Schenk
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Title: Method of Cryopreserving Selected Sperm Cells
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Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

PETITION FOR EXTENSION OF TIME UNDER 37 C.F.R. § 1.136(a)

On behalf of the applicant, the undersigned attorney hereby requests an extension of time pursuant to 37 C.F.R. § 1.136(a). This request is submitted in relation to a response due to the office action mailed February 9, 2006, having a shortened statutory period for response of three months. It is requested that this time period be extended for an additional three months to and including August 9, 2006. This extension will thereby encompass the statutory period of six months pursuant to 35 U.S.C. § 133. Pursuant to 37 C.F.R. § 1.17, an extension fee of \$1,020.00 for the third month of extension is included with this Petition and the accompanying response. In the event the fee has been inappropriately calculated, please allow the maximum extension possible and inform me at your earliest convenience so that any deficiency or error can be corrected immediately.

Dated this 9 day of August, 2006.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By: Nicole A. Ressue
Nicole A. Ressue
Attorney for Assignee
USPTO Reg. No. 48,665
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

08/14/2006 GWORDDF1 00000079 09478299

03 FC:1253

1020.00 DP



Express Mail No: EV 673965651 US

IN THE UNITED STATES PATENT AND
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Customer No.: 33549

CERTIFICATE OF EXPRESS MAILING

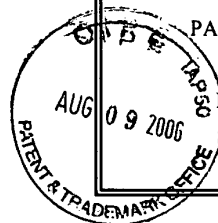
I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.
2. I have this day deposited the attached Petition for Extension of Time under 37 C.F.R. § 1.136(a) with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 9 day of August, 2006.

Cheryl A. Swanson
Cheryl A. Swanson

ExpressMailNo.EV 673965651 US

 IN THE UNITED STATES PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICATION NO:	09/478,299
	FILINGDATE:	1/5/2000
	FIRSTNAMEDINVENTOR	John L. Schenk
	ARTUNIT:	1654
	EXAMINERNAME:	M. Meller
	DOCKETNO:	22091-701

I. US PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO. & KIND CODE (if known)	PATENTEE OR APPLICANT NAME	PUBLICATION/ISSUE DATE	Pages, Columns, Lines Where Relevant Passages Or Relevant Drawings Appear
	4,559,309	Evenson	12/17/1985	
	4,999,283	Zavos et al.	3/12/1991	

II. FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL	Foreign Patent Document Country Code, Number, Kind Code (if known)	PATENTEE OR APPLICANT NAME	PUB'NDATE dd-yyyy	mm-	TRANSLATION Yes No

III. OTHER REFERENCES

EXAMINER INITIAL	Document
	Australian Application No. 17552/01; Monsanto Opposition, Statement of Grounds 03/06/2006
	European Appliction Number 00980567.9; English Translation of Opposition filed by European Union Parliament 11/5/2005 (Document originally filed in German.)
	European Appliction Number 00980567.9; English Translation of Opposition filed by Greenpeace 11/02/2005 (Document originally filed German.)
	New Zealand Application Number 530441, Examination Report dated 07/13/2005
	New Zealand Application Number 530441, Letters Patent Number 530441 dated 01/12/2006
	New Zealand Application Number 541086, Examination Report dated 07/13/2005
	Johnson, L. A., Sexing mammalian sperm for production of offspring: the state-of-the-art; Animal Reproduction Science; 60-61 (2000) pp 93-107
	Seidel, G.E. Jr., et al., Methods of Ovum Recovery and Factors Affecting Fertilization of Superovulated Bovine Ova, Control of Reproduction in the Cow, Sneenan ed., 1978, pp 268-280
	Hawk, H. W. et al., Effect of Unilateral Cornual Insemination upon Fertilization Rate in Superovulating and Single-Ovulating Cattle, Journal of Animal Sciences, 1986 vol. 63, pp 551-560
	Andersson, M. et al., Pregnancy Rates in Lactating Holstein-Greisian Cows after Artificial Insemination with Sexed Sperm. Reprod. Dom. Anim 41, 95-97, 2006
	Morton, K. M., et al., In vitro and in vivo survival of bisected sheep embryos derived from frozen-thawed unsorted, and frozen-thawed sex-sorted and refrozen-thawed ram spermatozoa; Theriogenology, 65 (2006) 1333-1345
	Wilson, R. D., et al., In vitro production of bovine embryos using sex-sorted sperm, Theriogenology, 65 (2006) 1007-1015
	Johnson, L.A., et al, 1996 Gender preselection in mammals. XX Beltsville Symposium in Agricultural Research Technolgy's Role in the Genetic Improvement of Farm Animals. pp. 151-164, Amer. Soc. Anim. Sci. IL, USA.
	Smorag, Z., et al., Cattle Sex Regulation by Separation of X and Y Spermatozoa – Preliminary Results of Field Experiment in Poland, Reproduction, Fertility and Development 17(2) 306–306; 01/01/2005
	Crichton, E., et al. (Abstract) Artificial Insemination of Lactating Holstein Cows with Sexed Sperm, Reproduction, Fertility and Development 18(2) 281 - 281, 12/14/2005
	Lindsey, A.C., et al. Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa, Equine Vet J. 2002 Mar;34(2):106-7.
	Drobnis, E. Z, Cold shock damage is due to lipid phase transitions in cell membranes : a demonstration using sperm as a model, Journal of experimental zoology (J. exp. zool.) 1993, vol. 265, no4, pp. 432-437 (22 ref.)
	Hagele, W.C., et al., Effect of Separating Bull Semen into X and Y Chromosome-bearing Fractions on the Sex Ratio of Resulting Embryos; Cran J. Comp. Med, 1984: 48:294-298
	US Patent Application Number 11/422,735 filed 05/25/2006; Apparatus, Methods and Processes for Sorting Particles and for Providing Sex-Sorted Animal Sperm
	Suh, T.K, et al., Pressure during flow sorting of bull sperm affects post-thaw motility characteristics; Theriogenology Vol. 59, No. 1, January 2003 p 516
Examiner	Date Considered
EXAMINER: Please initial if citation considered, whether or not citation is in conformance with MPEP § 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.	

Express Mail No: EV 673965651 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

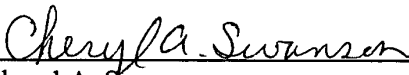
Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

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1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.
2. I have this day deposited the attached Information Disclosure Statement along with copies of Foreign Patent Documents and Other References cited with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 9 day of August, 2006.



Cheryl A. Swanson



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Attorney Docket: 22091-701
Customer No.: 33549

LETTER OF TRANSMITTAL

To: Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Enclosed for filing are the following documents:

1. a Petition for Extension of Time under 37 C.F.R. §1.136(a);
2. a Request for Continued Examination (RCE) Transmittal;
3. a Request for Continued Examination under 37 C.F.R. § 1.114;
4. an Information Disclosure Statement along with copies of Foreign Patent Documents and Other References cited;
5. this Letter of Transmittal along with a Credit Card Payment form authorized in the amount of \$1,910.00 (\$790.00 for the RCE fee, \$1,020.00 for the 3-month extension of time fee, and \$100.00 for 2 additional claims);

6. Certificates of Express Mailing for the above documents and a postcard receipt for return to the undersigned.

Please address all future correspondence to the address associated with Customer Number 33549 (Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, CO 80521).

Please confirm receipt of the documents by applying your date stamp on the enclosed postcard and returning it to me.

I have this 9 day of August, 2006, either myself personally or through my direction of staff at this office, deposited all of the items in the above letter of transmittal with the United States Postal Service as Express Mail, postage prepaid, in an envelope addressed to: Mail Stop RCE, Commissioner of Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 9 day of August, 2006.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By: Nicole A. Ressue
Nicole A. Ressue
Attorney for Assignee
USPTO Reg. No. 48,665
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

Express Mail No: EV 673965651 US

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

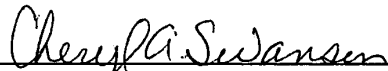
Application Number: 09/478,299
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Dated this 9 day of August, 2006.



Cheryl A. Swanson

PATENT APPLICATION FEE DETERMINATION RECORD
Effective October 1, 2003

Application or Docking Number:

09/478299

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)
TOTAL CLAIMS		
FOR	NUMBER FILED	NUMBER EXTRA
TOTAL CHARGEABLE CLAIMS	18 minus 20 =	-
INDEPENDENT CLAIMS	1 minus 3 =	-
MULTIPLE DEPENDENT CLAIM PRESENT ☐		

* If the difference in column 1 is less than zero, enter "0" in column 2

CLAIMS AS AMENDED - PART II

9-7-05

	(Column 1)	(Column 2)	(Column 3)
AMENDMENT A	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	13	Minus 20	=
Independent	1	Minus 3	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐			

5/24/06

	(Column 1)	(Column 2)	(Column 3)
AMENDMENT B	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	9	Minus 20	=
Independent	1	Minus 3	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐			

5/24/06

	(Column 1)	(Column 2)	(Column 3)
AMENDMENT C	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	3.3	Minus 3.4	= 0
Independent	0	Minus 3	= 0
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐			

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

SMALL ENTITY TYPE ☐

OR OTHER THAN SMALL ENTITY

RATE	FEE
BASIC FEE	340
XS 9=	
X43=	
+145=	
TOTAL	340

RATE	FEE
BASIC FEE	
XS16=	
X86=	
+290=	
TOTAL	

SMALL ENTITY OR

OTHER THAN SMALL ENTITY

RATE	ADDITIONAL FEE
XS 9=	
X43=	
+145=	
TOTAL ADDIT. FEE	

RATE	ADDITIONAL FEE
XS18=	
X86=	
+290=	
TOTAL ADDIT. FEE	

RATE	ADDITIONAL FEE
XS 9=	
X43=	
+145=	
TOTAL ADDIT. FEE	

RATE	ADDITIONAL FEE
XS18=	
X86=	
+290=	
TOTAL ADDIT. FEE	

RATE	ADDITIONAL FEE
XS 9=	
X43=	
+145=	
TOTAL ADDIT. FEE	

RATE	ADDITIONAL FEE
XS18=	
X86=	
+290=	
TOTAL ADDIT. FEE	

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

**FIRST SUPPLEMENT TO
REQUEST FOR CONTINUED EXAMINATION OF AUGUST 9, 2006**

This submission is a First Supplement to the Request for Continued Examination filed in the above-referenced case on August 9, 2006. The Request for Continued Examination was filed pursuant to 37 C.F.R. § 1.114 in response to an Office Action dated February 9, 2006, and was filed with an appropriate petition for extension of time. In addition, this First Supplement to the Request for Continued Examination is filed in response to the personal interview held on September 18, 2006 between Examiner Meller and Mr. Santangelo.

This First Supplement is submitted to provide data and certification of data by John L. Schenk and a supplemental information disclosure statement.

Although not raised in the action, the following may be noteworthy. Salisbury, G. W. and VanDemark, N. L. "Physiology of Reproduction and Artificial Insemination of Cattle." San Francisco: Freeman and Company. p. 442-551 (1978), Chapters 16 and 17, herein referred to as "Salisbury" and US Pat. No. 5,021,244 to Spaulding, herein referred to as "Spaulding" does not anticipate nor make obvious the claims of the present application. Page 477 of the Spaulding reference discusses extended samples to 5, 10, and 15 million motile cells per milliliter. This discussion does not explicitly nor implicitly state that these extended samples use sperm cells that have been sorted and even that the extended samples will be cooled and frozen.

Accordingly, the reference does not disclose, *inter alia*, sorting of sperm cells, cooling the sex-selected sperm cells, suspending sperm cells in an extender to about 5 million per milliliter of extender to about 10 million per milliliter of extender, freezing the sex-selected sperm cells in an extender, and as further discussed in the claims of the present application.

In addition, the Spaulding reference, alone or perhaps even in combination with the Salisbury reference, does not disclose or make obvious the invention as claimed. The Spaulding reference does not disclose, *inter alia*, sorting of sperm cells using flow cytometry or fluorescence-activated cell sorting, suspending the sex-selected sperm cells in an extender to about 5 million per milliliter of extender to about 10 million per milliliter of extender and freezing the sex-selected sperm cells in an extender, and as further discussed in the claims of the present application. To reiterate, as one example, the Spaulding reference does not discuss freezing of sperm cells that have been sorted by flow cytometry or fluorescence-activated cell sorting of which the sperm cells have been suspended in about 5 million per milliliter of extender to about 10 million per milliliter of extender. Further, since the Salisbury and Spaulding references do not disclose all of the elements of the claimed invention, they do not anticipate nor make obvious the claimed invention.

In further support, it is noted that the Affidavit by John L. Schenk filed on January 20, 2006 discusses how the Spaulding and Shrimpton references are not capable of being successfully reproduced. Accordingly, the Affidavit asserts that the references are inoperable by a preponderance of the evidence by demonstrating that one skilled in the art would have been unable to successfully produce the techniques as discussed in the Spaulding and Shrimpton references. *See, In re Sasse*, 629 F.2d 675, 207, USPQ 107 (CCPA 1980).

Dated this 2nd day of October, 2006.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By: /Nicole A. Ressue/
Nicole A. Ressue
Attorney for Assignee
USPTO Reg. No. 48,665
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

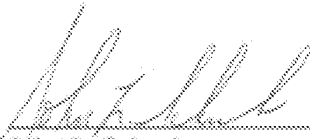
IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

CERTIFICATION

I, John L. Schenk, certify that the attached data regarding the effect of dilution on 5°C cooled sperm was documented and observed by myself in August 2006. The objective of this experiment was to accomplish continuity data for sperm motility at dilution concentrations between 5×10^6 sperm/ml and 10×10^6 sperm/ml.

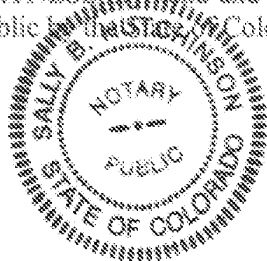
Dated this 22 day of September, 2006.

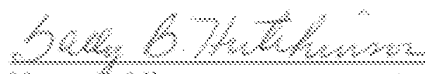

John L. Schenk

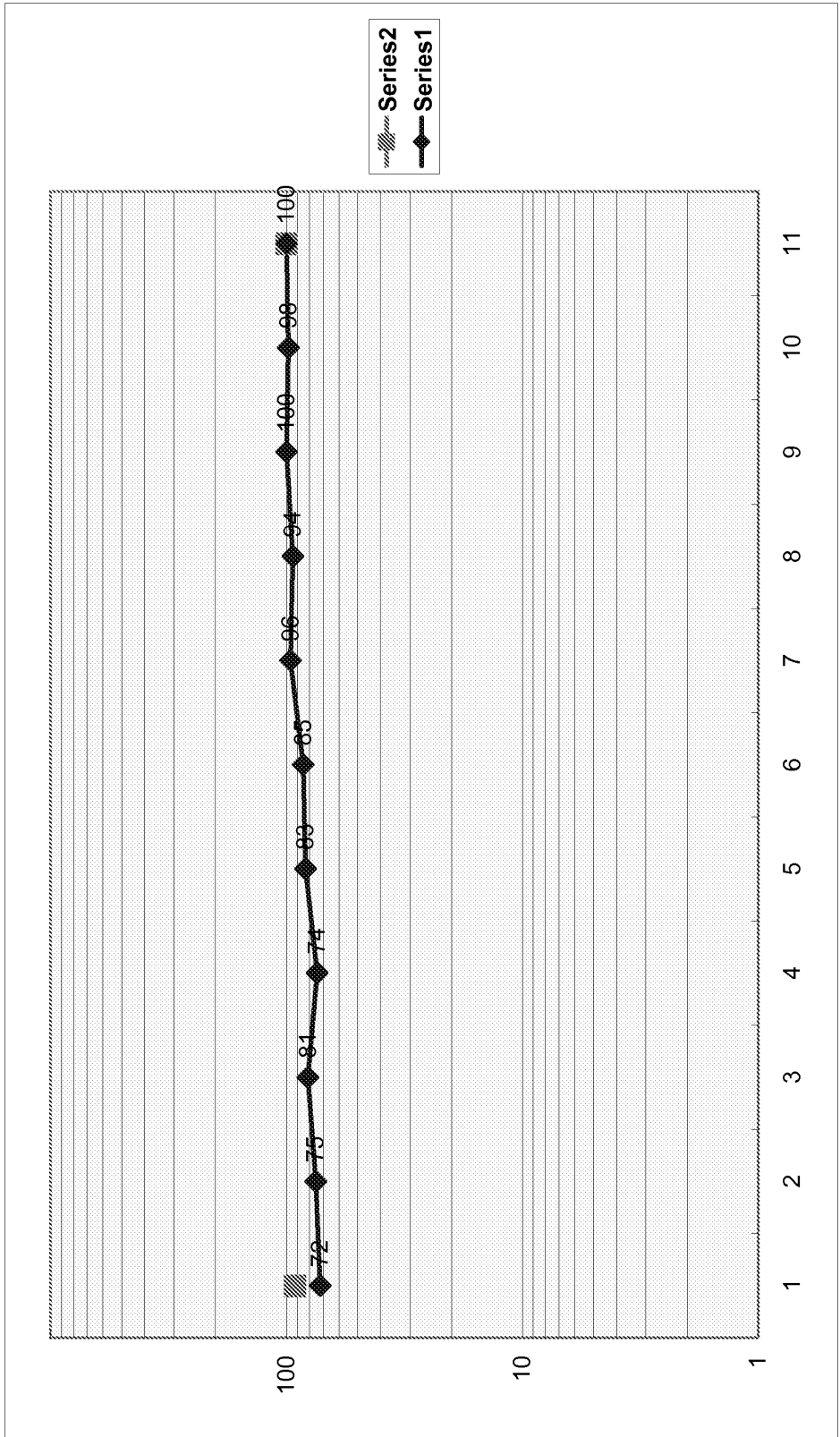
UNITED STATES OF AMERICA)
STATE OF COLORADO)ss.
COUNTY OF LARIMER)

SUBSCRIBED AND SWORN to before me in the County of Larimer, State of Colorado, United States of America, by John L. Schenk, this 22 day of September, 2006.

WITNESS my hand and official seal pursuant to the authority vested in me as a Notary Public in the State of Colorado.




Notary Public
My Commission Expires: 9/18/2009



Bull	Sperm/ml	John 24h	Kathy 24h
HO040	5.00	30	35
HO040	5.50	30	35
HO040	6.00	30	45
HO040	6.50	40	40
HO040	7.00	45	45
HO040	7.50	45	40
HO040	8.00	45	45
HO040	8.50	45	45
HO040	9.00	50	50
HO040	9.50	45	45
HO040	10.00	50	45
HO047	5.00	20	35
HO047	5.50	25	35
HO047	6.00	25	35
HO047	6.50	20	25
HO047	7.00	20	35
HO047	7.50	25	35
HO047	8.00	35	40
HO047	8.50	30	40
HO047	9.00	35	40
HO047	9.50	35	35
HO047	10.00	40	30
HO048	5.00	30	40
HO048	5.50	30	45
HO048	6.00	35	45
HO048	6.50	30	40
HO048	7.00	35	40
HO048	7.50	35	45
HO048	8.00	45	45
HO048	8.50	45	45
HO048	9.00	45	45
HO048	9.50	50	50
HO048	10.00	55	45

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)	Application Number		09478299	
	Filing Date		2000-01-05	
	First Named Inventor	John L. Schenk		
	Art Unit	1654		
	Examiner Name	M. Meller		
	Attorney Docket Number	22091-701		

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Examiner Initial*	Cite No	Patent Number	Kind Code ¹	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear		
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	2	7094527		2006-08-22	Seidel, et al.			
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Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear		
	1	20050244805	A1	2005-11-03	Ludwig, et al.			
	2	20050282245		2005-12-22	Ludwig et al.			
	3	20050214733	A1	2005-09-29	Graham et al.			
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)	Application Number		09478299	
	Filing Date		2000-01-05	
	First Named Inventor	John L. Schenk		
	Art Unit	1654		
	Examiner Name	M. Meller		
	Attorney Docket Number	22091-701		

	1	93/17322	WO	A1	1993-09-02	Univ. of Hertfordshire GB		☐
	2	2006012597	WO	A2	2006-02-02	Monsanto Technology LLC		☐
	3	1471019	UK		1977-04-21	United Aircraft Corp.		☐

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NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T ⁵
	1	US Nonprovisional Application Number 10/266,562, Filed 10/07/2002; Office Action Dated 08/23/2006	☐
	2	Rath, D, et al., In Vitro Production of Sexed Embryos for Gender Preselection: High-speed sorting of X-Chromosome-Bearing Sperm to Produce Pigs After Embryo Transfer, J. Anim. Sci. 1999, 77:3346-3352	☐
	3	Auchtung, T.L., et al., Effects of Photoperiod During the Dry Period on Prolactin, Prolactin Receptor, and Milk Production of Dairy Cows; Journal of Dairy Sci. 88: 121-127; American Dairy Sci. Assoc., 2005.	☐
	4	Bailey, Tom and Currin, John Milk Production Evaluation In First Lactation Heifers; 1999 Virginia Cooperation Extension/Dairy Science Publication 404-285	☐
	5	Belloin, J.C., Milk and Dairy products: production and processing costs Food and Agriculture Organization of United Nations Rome 1988 FAO; web page where found: www.fao.org/docrep/003/x6931e/X6931E00.htm	☐
	6	Kume, Shin-ichi; Dept of Animal Nutrition National Institute of Animal Industry Tsukuba 305, Japan THE DAIRY INDUSTRY \$IN ASIA B. JAPAN; www.agnet.org/library/article/eb384b.html	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)	Application Number		09478299
	Filing Date		2000-01-05
	First Named Inventor	John L. Schenk	
	Art Unit	1654	
	Examiner Name	M. Meller	
	Attorney Docket Number	22091-701	

7	Crichton, E.; Huffman, S.; McSweeney, K.; and Schenk, J. 347 Artificial Insemination of Lactating Holstein Cows with sexed sperm: Abstract CSORP Publishing - Reproduction, Fertility and Development www.publish.csiro.au/nid/44/paper/RDv18n2Ab347.htm	☐
8	Lopez, H., Caraviello, D.Z., Satter, L.D., Fricke, P.M. and Wiltbank, M.C.; Relationship Between Level of Milk Production and Multiple Ovulation in Lactating Dairy Cows Journal of Dairy Sci. 88:2783-2793; American Dairy Science Association, 2005.	☐
9	Managing the Dairy Cow During the Dry Period; Dairy Cattle Production 341-450A; Macdonald Campus of McGill University/Faculty of Agricultural & Environmental Sciences/Department of Animal Science	☐
10	Milk Production and Biosynthesis University of Guelph/Dairy Science and Technology www.foodsci.uoguelph.ca/dairyedu/biosyntheses.html	☐
11	MILK PRODUCTION Released 7-18-2006, by the National Agricultural Statistics Service (NASS), Agri. Stats. Board, US Dept of Agri.	☐
12	De Vries, A. Economic Value of Pregnancy in Dairy Cattle Journal of Dairy Sci. 89:3876-3885/American Dairy Sci. Assoc. 2006	☐

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

¹ See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. ² Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). ³ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁴ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁵ Applicant is to place a check mark here if English language translation is attached.

**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**
(Not for submission under 37 CFR 1.99)

Application Number	09478299
Filing Date	2000-01-05
First Named Inventor	John L. Schenk
Art Unit	1654
Examiner Name	M. Meller
Attorney Docket Number	22091-701

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ None

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Nicole A. Ressue/	Date (YYYY-MM-DD)	2006-10-02
Name/Print	Nicole A. Ressue	Registration Number	48,665

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	1231303
Application Number:	09478299
Confirmation Number:	1509
Title of Invention:	Method Of Cryopreserving Selected Sperm Cells
First Named Inventor:	John L. Schenk
Correspondence Address:	Craig Miles Santangelo Law Offices P C 125 South Howes Street 3rd Floor Fort Collins CO 80521 US 650-849-4800 -
Filer:	Nicole Anderson Ressue/Cheryl A. Swanson
Filer Authorized By:	Nicole Anderson Ressue
Attorney Docket Number:	22091-701
Receipt Date:	02-OCT-2006
Filing Date:	05-JAN-2000
Time Stamp:	18:02:01
Application Type:	Utility
International Application Number:	

Payment information:

Submitted with Payment	no

File Listing:

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Warnings:					
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3	Supplemental Response or Supplemental Amendment	Certification_of_inventor_Sc henk.pdf	841376	no	3
Warnings:					
Information:					
4	Information Disclosure Statement (IDS) Filed	Frozen_299_IDS_Form.pdf	1410447	no	5
Warnings:					
Information:					
5		Foreign_References_cited.pdf	4606373	yes	95
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	Foreign Reference		1	34	
	Foreign Reference		35	43	
	Foreign Reference		44	95	
Warnings:					
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6		Other_Non_Patent_References.pdf	5941690	yes	94
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IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

LETTER OF TRANSMITTAL

Filed via EFS this same date for filing are the following documents:

1. First Supplement to Request for Continued Examination of August 9, 2006;
2. a Certification of John L. Schenk along with a graphical depiction of the data described as well as the data points;
3. an Information Disclosure Statement along with a copy of each Foreign Patent Document or Non-Patent Literature Document listed; and
4. this Letter of Transmittal.

Please address all future correspondence to the address associated with Customer Number 33549 (Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, CO 80521.)

Dated this 2nd day of October, 2006.

Respectfully Submitted,
SANTANGELO LAW OFFICES, P.C.
By: /Nicole A. Ressue/
Nicole A. Ressue
Attorney for Assignee
PTO No. 48,665
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

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<u>L3</u>	freez\$5 or cryopreser\$5	332139	<u>L3</u>
<u>L2</u>	sex	66365	<u>L2</u>
<u>L1</u>	sperm	35992	<u>L1</u>

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NOTICE OF ALLOWANCE AND FEE(S) DUE

7590 12/05/2006

Craig Miles
Santangelo Law Offices P C
125 South Howes Street
3rd Floor
Fort Collins, CO 80521

EXAMINER

MELLER, MICHAEL V

ART UNIT

PAPER NUMBER

1655

DATE MAILED: 12/05/2006

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509
TITLE OF INVENTION: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS				

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	\$700	\$0	\$0	\$700	03/05/2007

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

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If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

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- B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

- A. Pay TOTAL FEE(S) DUE shown above, or
- B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

**Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885**

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

7590 12/05/2006

Craig Miles
Santangelo Law Offices P C
125 South Howes Street
3rd Floor
Fort Collins, CO 80521

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509

TITLE OF INVENTION: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	\$700	\$0	\$0	\$700	03/05/2007

EXAMINER	ART UNIT	CLASS-SUBCLASS
MELLER, MICHAEL V	1655	435-001100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
 (1) the names of up to 3 registered patent attorneys or agents OR, alternatively,
 (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
 1 _____
 2 _____
 3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

- ☐ Issue Fee
☐ Publication Fee (No small entity discount permitted)
☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): (Please first reapply any previously paid issue fee shown above)

- ☐ A check is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. Change in Entity Status (from status indicated above)

- ☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27. ☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____

Date _____

Typed or printed name _____

Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509
7590		12/05/2006	EXAMINER	
Craig Miles		MELLER, MICHAEL V		
Santangelo Law Offices P C		ART UNIT		
125 South Howes Street		PAPER NUMBER		
3rd Floor		1655		
Fort Collins, CO 80521		DATE MAILED: 12/05/2006		

Determination of Patent Term Extension under 35 U.S.C. 154 (b) (application filed after June 7, 1995 but prior to May 29, 2000)

The Patent Term Extension is 0 day(s). Any patent to issue from the above-identified application will include an indication of the 0 day extension on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Extension is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Notice of Allowability	Application No.	Applicant(s)	
	09/478,299	SCHENK, JOHN L.	
	Examiner	Art Unit	
	Michael V. Meller	1655	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to the amendment filed 8/9/2006.
2. ☒ The allowed claim(s) is/are 38-64 and 67.
3. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
 - a) ☐ All b) ☐ Some* c) ☐ None of the:
 1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

* Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

4. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
 - (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
 - 1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
 - (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892) 2. ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948) 3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____ 4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material	5. ☐ Notice of Informal Patent Application 6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ 7. ☒ Examiner's Amendment/Comment 8. ☒ Examiner's Statement of Reasons for Allowance 9. ☐ Other _____

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Nicole Ressue on 10/10/2006.

The application has been amended as follows:

IN THE CLAIMS:

Claim 38, line 4, delete, "create" and insert, ---provide---, line 5, insert, ---obtained--- before "using flow cytometry", line 7, delete, "to about 5 million" and insert, ---to provide a concentration of sperm cells of about 5 million---.

Claims 40, 41, 43, 44 delete "and" in line 1, in line 2, delete "the step" and insert - --a step---.

Claim 45, line 2, delete, "the temperature" and insert, ---temperature---.

Claim 57, lines 1-2, delete, "claim 47, 51, 53 or 55" and insert, ---claim 47---.

Art Unit: 1655

Delete claims 65, 68, 69.

Claims 40, 41, 55, 56, 60, 61, 62, 67 were withdrawn and are now hereby re-introduced as examined and pending and allowed claims.

The following is an examiner's statement of reasons for allowance: the declaration of Dr. Schenk filed January 20, 2006, proves that the references were inoperable and thus cannot be used in a 35 USC 103 rejection against the claims.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 571-272-0967. The examiner can normally be reached on Monday thru Thursday: 9:30am-6:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Terry McKelvey can be reached on 571-272-0775. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Art Unit: 1655

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.


Michael V. Meller
Primary Examiner
Art Unit 1655

MVM

Interview Summary	Application No.		Applicant(s)	
	09/478,299		SCHENK, JOHN L.	
	Examiner		Art Unit	
	Michael V. Meller		1655	

All participants (applicant, applicant's representative, PTO personnel):

- (1) Michael V. Meller. (3) _____
 (2) Luke Santangelo. (4) _____

Date of Interview: 18 September 2006.

Type: a) ☐ Telephonic b) ☐ Video Conference
 c) ☒ Personal [copy given to: 1) ☒ applicant 2) ☐ applicant's representative]

Exhibit shown or demonstration conducted: d) ☐ Yes e) ☒ No.
 If Yes, brief description: _____.

Claim(s) discussed: All of record.

Identification of prior art discussed: All of record.

Agreement with respect to the claims f) ☐ was reached. g) ☒ was not reached. h) ☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: The applicant raised the issue of the references being allegedly inoperable as was stated in the declaration. Applicant will submit further data on the test points from about 5 million to about 10 million for the final extender The examiner at that time will revisit the claims and update the search and consider possible favorable claims amendments..

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.



Examiner's signature, if required

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record

A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.

All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:

- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

- 1) A brief description of the nature of any exhibit shown or any demonstration conducted,
- 2) an identification of the claims discussed,
- 3) an identification of the specific prior art discussed,
- 4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
- 5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
- 6) a general indication of any other pertinent matters discussed, and
- 7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.



Express Mail No: EV 419716875 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT	DOCKET NO. XY Frozen RCE	Serial No. - 09/478,299
	APPLICANT (S) John L. Schenk	
	FILING DATE January 5, 2000	ART UNIT - 1654

I. U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO	DATE	NAME	CLASS	SUBCLASS	FILING DATE
<i>MTM</i>	6,395,305 B1	05/08/2002	Buhr et al.	424	520	09/22/1998

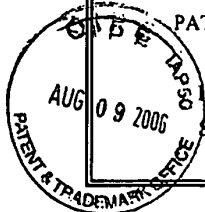
II. FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						Yes	No

III. OTHER DOCUMENTS

EXAMINER	<i>[Signature]</i>	DATE CONSIDERED	<i>10/16/06</i>
EXAMINER: Please initial if citation considered, whether or not citation is in conformance with MPEP § 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.			

ExpressMailNo.EV 673965651 US



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICATION NO:	09/478,299
	FILINGDATE:	1/5/2000
	FIRSTNAMEDINVENTO	John L. Schenk
	ARTUNIT:	1654
	EXAMINERNAME:	M. Meller
	DOCKETNO:	22091-701

I. US PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NO. & KIND CODE (if known)	PATENTEE OR APPLICANT NAME	PUBLICATION/ISSUE DATE	Pages, Columns, Lines Where Relevant Passages Or Relevant Drawings Appear
<i>MM</i>	4,559,309	Evenson	12/17/1985	
<i>MM</i>	4,999,283	Zavos et al.	3/12/1991	

II. FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL	Foreign Patent Document Country Code, Number, Kind Code (if known)	PATENTEE OR APPLICANT NAME	PUB'NDATE dd-yyyy	TRANSLATION Yes No

III. OTHER REFERENCES

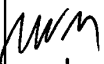
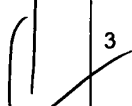
EXAMINER INITIAL	Document
MM	Australian Application No. 17552/01; Monsanto Opposition, Statement of Grounds 03/06/2006
	European Application Number 00980567.9; English Translation of Opposition filed by European Union Parliament 11/5/2005 (Document originally filed in German.)
	European Application Number 00980567.9; English Translation of Opposition filed by Greenpeace 11/02/2005 (Document originally filed German.)
	New Zealand Application Number 530441, Examination Report dated 07/13/2005
	New Zealand Application Number 530441, Letters Patent Number 530441 dated 01/12/2006
	New Zealand Application Number 541086, Examination Report dated 07/13/2005
	Johnson, L. A., Sexing mammalian sperm for production of offspring: the state-of-the-art; Animal Reproduction Science; 60-61 (2000) pp 93-107
	Seidel, G.E. Jr., et al., Methods of Ovum Recovery and Factors Affecting Fertilization of Superovulated Bovine Ova, Control of Reproduction in the Cow, Sneenan ed., 1978, pp 268-280
	Hawk, H. W. et al., Effect of Unilateral Cornual Insemination upon Fertilization Rate in Superovulating and Single-Ovulating Cattle, Journal of Animal Sciences, 1986 vol. 63, pp 551-560
	Andersson, M. et al., Pregnancy Rates in Lactating Holstein-Friesian Cows after Artificial Insemination with Sexed Sperm. Reprod. Dom. Anim 41, 95-97, 2006
	Morton, K. M., et al., In vitro and in vivo survival of bisected sheep embryos derived from frozen-thawed unsorted, and frozen-thawed sex-sorted and refrozen-thawed ram spermatozoa; Theriogenology, 65 (2006) 1333-1345
	Wilson, R. D., et al., In vitro production of bovine embryos using sex-sorted sperm, Theriogenology, 65 (2006) 1007-1015
	Johnson, L.A., et al, 1996 Gender preselection in mammals. XX Beltsville Symposium in Agricultural Research Technology's Role in the Genetic Improvement of Farm Animals. pp. 151-164, Amer. Soc. Anim. Sci. IL, USA.
	Smorag, Z., et al., Cattle Sex Regulation by Separation of X and Y Spermatozoa - Preliminary Results of Field Experiment in Poland, Reproduction, Fertility and Development 17(2) 306-306; 01/01/2005
	Crichton, E., et al. (Abstract) Artificial Insemination of Lactating Holstein Cows with Sexed Sperm, Reproduction, Fertility and Development 18(2) 281 - 281, 12/14/2005
	Lindsey, A.C., et al. Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa, Equine Vet J. 2002 Mar;34(2):106-7.
	Drobnis, E. Z, Cold shock damage is due to lipid phase transitions in cell membranes : a demonstration using sperm as a model, Journal of experimental zoology (J. exp. zool.) 1993, vol. 265, no4, pp. 432-437 (22 ref.)
	Hagele, W.C., et al., Effect of Separating Bull Semen into X and Y Chromosome-bearing Fractions on the Sex Ratio of Resulting Embryos; Cran J. Comp. Med, 1984: 48:294-298
	US Patent Application Number 11/422,735 filed 05/25/2006; Apparatus, Methods and Processes for Sorting Particles and for Providing Sex-Sorted Animal Sperm
	Suh, T.K, et al., Pressure during flow sorting of bull sperm affects post-thaw motility characteristics; Theriogenology Vol. 59, No. 1, January 2003 p 516
Examiner	Date Considered 10/10/06
EXAMINER: Please initial if citation considered, whether or not citation is in conformance with MPEP § 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.	

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)	Application Number	09478299
	Filing Date	2000-01-05
	First Named Inventor	John L. Schenk
	Art Unit	1654
	Examiner Name	M. Meller
	Attorney Docket Number	22091-701

U.S. PATENTS							Remove	
Examiner Initial*	Cite No	Patent Number	Kind Code ¹	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear		
	1	4007087		1977-02-08	Ericsson			
	2	7094527		2006-08-22	Seidel, et al.			
If you wish to add additional U.S. Patent citation information please click the Add button.							Add	
U.S. PATENT APPLICATION PUBLICATIONS							Remove	
Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear		
	1	20050244805	A1	2005-11-03	Ludwig, et al.			
	2	20050282245		2005-12-22	Ludwig et al.			
	3	20050214733	A1	2005-09-29	Graham et al.			
If you wish to add additional U.S. Published Application citation information please click the Add button.							Add	
FOREIGN PATENT DOCUMENTS							Remove	
Examiner Initial*	Cite No	Foreign Document Number ³	Country Code ² i	Kind Code ⁴	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T ⁵

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)	Application Number		09478299	
	Filing Date		2000-01-05	
	First Named Inventor		John L. Schenk	
	Art Unit		1654	
	Examiner Name		M. Meller	
	Attorney Docket Number		22091-701	

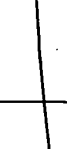
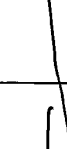
	1	93/17322	WO	A1	1993-09-02	Univ. of Hertfordshire GB	☐
	2	2006012597	WO	A2	2006-02-02	Monsanto Technology LLC	☐
	3	1471019	UK		1977-04-21	United Aircraft Corp.	☐

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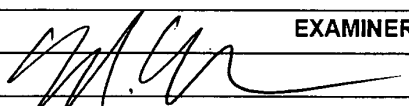
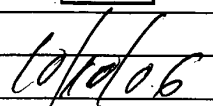
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NON-PATENT LITERATURE DOCUMENTS

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T ⁵
	1	US Nonprovisional Application Number 10/266,562, Filed 10/07/2002; Office Action Dated 08/23/2006	☐
	2	Rath, D, et al., In Vitro Production of Sexed Embryos for Gender Preselection: High-speed sorting of X-Chromosome-Bearing Sperm to Produce Pigs After Embryo Transfer, J. Anim. Sci. 1999, 77:3346-3352	☐
	3	Auchtung, T.L., et al., Effects of Photoperiod During the Dry Period on Prolactin, Prolactin Receptor, and Milk Production of Dairy Cows; Journal of Dairy Sci. 88: 121-127; American Dairy Sci. Assoc., 2005.	☐
	4	Bailey, Tom and Currin, John Milk Production Evaluation In First Lactation Heifers; 1999 Virginia Cooperation Extension/Dairy Science Publication 404-285	☐
	5	Belloin, J.C., Milk and Dairy products: prduction and processing costs Food and Agriculture Organization of United Nations Rome 1988 FAO; web page where found: www.fao.org/docrep/003/x6931e/X6931E00.htm	☐
	6	Kume, Shin-ichi; Dept of Animal Nutrition National Institute of Animal Industry Tsukuba 305, Japan THE DAIRY INDUSTRY \$IN ASIA B. JAPAN; www.agnet.org/library/article/eb384b.html	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)	Application Number		09478299
	Filing Date		2000-01-05
	First Named Inventor	John L. Schenk	
	Art Unit	1654	
	Examiner Name	M. Meller	
	Attorney Docket Number	22091-701	

	7	Crichton, E.; Huffman, S.; McSweeney, K.; and Schenk, J. 347 Artificial Insemination of Lactating Holstein Cows with sexed sperm: Abstract CSORP Publishing - Reproduction, Fertility and Development www.publish.csiro.au/nid/44/paper/RDv18n2Ab347.htm	☐
	8	Lopez, H., Caraviello, D.Z., Satter, L.D., Fricke, P.M. and Wiltbank, M.C.; Relationship Between Level of Milk Production and Multiple Ovulation in Lactating Dairy Cows Journal of Dairy Sci. 88:2783-2793; American Dairy Science Association, 2005.	☐
	9	Managing the Dairy Cow During the Dry Period; Dairy Cattle Production 341-450A; Macdonald Campus of McGill University/Faculty of Agricultural & Environmental Sciences/Department of Animal Science	☐
	10	Milk Production and Biosynthesis University of Guelph/Dairy Science and Technology www.foodsci.uoguelph.ca/dairyedu/biosyntheses.html	☐
	11	MILK PRODUCTION Released 7-18-2006, by the National Agricultural Statistics Service (NASS), Agri. Stats. Board, US Dept of Agri.	☐
	12	De Vries, A. Economic Value of Pregnancy in Dairy Cattle Journal of Dairy Sci. 89:3876-3885/American Dairy Sci. Assoc. 2006	☐
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[illegible]

Michael V. Meller

1655

[illegible][illegible]

Class	Subclass	Date	Examiner
PG Pub	Search	10/10/06	WV



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Bib Data Sheet

CONFIRMATION NO. 1509

SERIAL NUMBER 09/478,299	FILING OR 371(c) DATE 01/05/2000 RULE	CLASS 435	GROUP ART UNIT 1655	ATTORNEY DOCKET NO. 22091-701
APPLICANTS John L. Schenk, Fort Collins, CO; ** CONTINUING DATA ***** This appln claims benefit of 60/167,423 11/24/1999 ** FOREIGN APPLICATIONS *****				
IF REQUIRED, FOREIGN FILING LICENSE GRANTED** SMALL ENTITY ** ** 02/28/2000				
Foreign Priority claimed ☐ yes ☒ no 35 USC 119 (a-d) conditions ☐ yes ☒ no ☐ Met after met Verified and Acknowledged <u> </u> <u> </u> Examiner's Signature Initials		STATE OR COUNTRY CO	SHEETS DRAWING	TOTAL CLAIMS 37 INDEPENDENT CLAIMS 2
ADDRESS Craig Miles Santangelo Law Offices P C 125 South Howes Street 3rd Floor Fort Collins, CO80521				
TITLE Method Of Cryopreserving Selected Sperm Cells				
FILING FEE RECEIVED 598	FEES: Authority has been given in Paper No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:		☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit	

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
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INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

7590 12/05/2006

Craig Miles
Santangelo Law Offices P C
125 South Howes Street
3rd Floor
Fort Collins, CO 80521

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I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509

TITLE OF INVENTION: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	No	\$1400.00	\$0	\$0	\$700	03/05/2007

EXAMINER	ART UNIT	CLASS-SUBCLASS
MELLER, MICHAEL V	1655	435-001100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. Use of a Customer Number is required.

2. For printing on the patent front page, list

- (1) the names of up to 3 registered patent attorneys or agents OR, alternatively,
- (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

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(A) NAME OF ASSIGNEE

XY, Inc.

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Fort Collins, CO 80524

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

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- ☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27. ☒ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

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Authorized Signature /Nicole A. Ressue/

Date December 7, 2006

Typed or printed name Nicole A. Ressue

Registration No. 48,665

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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Electronic Patent Application Fee Transmittal				
Application Number:		09478299		
Filing Date:		05-Jan-2000		
Title of Invention:		METHOD OF CRYOPRESERVING SELECTED SPERM CELLS		
First Named Inventor/Applicant Name:		John L. Schenk		
Filer:		Nicole Anderson Ressue/Cheryl A. Swanson		
Attorney Docket Number:		22091-701		
Filed as Large Entity				
Utility Filing Fees				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl issue fee	1501	1	1400	1400
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
Total in USD (\$)				1400

Electronic Acknowledgement Receipt	
EFS ID:	1359922
Application Number:	09478299
International Application Number:	
Confirmation Number:	1509
Title of Invention:	METHOD OF CRYOPRESERVING SELECTED SPERM CELLS
First Named Inventor/Applicant Name:	John L. Schenk
Correspondence Address:	Craig Miles Santangelo Law Offices P C 125 South Howes Street 3rd Floor Fort Collins CO 80521 US 650-849-4800 -
Filer:	Nicole Anderson Ressue/Cheryl A. Swanson
Filer Authorized By:	Nicole Anderson Ressue
Attorney Docket Number:	22091-701
Receipt Date:	07-DEC-2006
Filing Date:	05-JAN-2000
Time Stamp:	17:51:10
Application Type:	Utility

Payment information:

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	EFS-xmt.pdf	80995	no	1
Warnings:					
Information:					
2	Miscellaneous Incoming Letter	Notification_of_change_Entity_Status.pdf	126232	no	1
Warnings:					
Information:					
3	Issue Fee Payment Recorded	Part_B_Fees_Transmital.pdf	2990859	no	1
Warnings:					
Information:					
4	Fee Worksheet (PTO-875)	fee-info.pdf	8162	no	2
Warnings:					
Information:					
Total Files Size (in bytes):			3206248		
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IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

Application Number: 09/478,299
Applicants: John L. Schenk
Filed: January 5, 2000
Title: Method of Cryopreserving Selected Sperm Cells
Group Art Unit: 1654
Examiner: M. Meller
Assignee: XY, Inc.
Attorney Docket: 22091-701
Customer No.: 33549

LETTER OF TRANSMITTAL

The following documents were filed via EFS this same date:

1. a true and correct copy of the Notification of Change of Small Entity Status filed October 17, 2003;
2. Part B – Fee(s) Transmittal;
3. this Letter of Transmittal;
4. an Electronic Patent Application Fee Transmittal showing a payment of \$1,400.00, the large entity fee for an Issue Fee – this fee is being paid at the time of this filing via Credit Card.

Please address all future correspondence to the address associated with Customer Number 33549 (Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, CO 80521.)

Dated this 7th day of December, 2006.

Respectfully Submitted,
SANTANGELO LAW OFFICES, P.C.

By: /Nicole A. Ressue/
Nicole A. Ressue
Attorney for Assignee
PTO No. 48,665
125 South Howes, Third Floor
Fort Collins, Colorado 80521
(970) 224-3100

Facsimile Number: 703-872-9306

Attorney Docket: 22091-701

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

n Re the Application of: John L. Schenk

Serial Number: 09/478,299

Filed: January 5, 2000

Parent Title: Method of Cryopreserving Selected Sperm Cells

Group Art Unit: 1654

Examiner: M. Meller

Assignee: XY, Inc.

NOTIFICATION OF CHANGE OF SMALL ENTITY STATUS

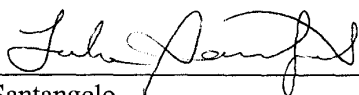
Dear Sir:

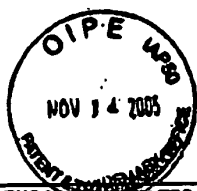
Pursuant to 37 C.F.R. 1.27(g)2 this document hereby notifies the Office that small entity status as previously established in the above referenced matter is no longer appropriate.

This notification of change in status is filed prior to or at the time of paying any issue, maintenance, or other fees after the change in status occurred. As required by the rule, this notification is signed by the attorney of record acting in a representative capacity pursuant to 37 C.F.R. 1.27(g)2.


Dated this 17 day of October, 2003.

Respectfully Submitted,
SANTANGELO LAW OFFICES, P.C.

By: 
Luke Santangelo
USPTO Reg. No. 31,997
125 South Howes, 3rd Floor
Fort Collins, Colorado 80521
(970)224-3100



ExpressMailNo.EV603557196US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICATIONNO:	09/478,299
	FILINGDATE:	1/5/2000
	FIRSTNAMEDINVENTO	John L. Schenk
	ARTUNIT:	~~4864~~ 1655.
	EXAMINERNAME:	M. Meller
	DOCKETNO:	22091-701

I. US PATENT DOCUMENTS

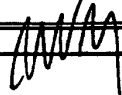
EXAMINER INITIAL	DOCUMENT NO. & KIND CODE (if known)	PATENTEE OR APPLICANT	PUBLICATION/ISSUE DATE	Pages, Columns, Lines Where Relevant Passages Or Relevant Drawings Appear
AMM	2002/0113965 A1	Roche et al.	8/22/2002	
	2002/0119558 A1	Seidel et al.	8/29/2002	
	2002/141902 A1	Asbury et al.	10/3/2002	
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		2004/0031071 A1	Morris et al.	2/12/2004
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ewc

2/16

09/478, 299

II. FOREIGN PATENT DOCUMENTS

EXAMINER INITIAL	Foreign Patent Document Country Code, Number, Kind Code (if known)	PATENTEE OR APPLICANT NAME	PUB'NDATE mm-dd-yyyy	TRANSLATION	
				Yes	No
	BR 9704313	Alves, E.	8/4/1999		
		GSF - Forschungszentrum			
	DE 195 49 015 C1		4/3/1997		
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cwc 2/16/07

09/478,299

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	04/24/2007	7208265	22091-701	1509

7590 04/04/2007

Craig Miles
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125 South Howes Street
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Fort Collins, CO 80521

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Extension under 35 U.S.C. 154 (b) (application filed after June 7, 1995 but prior to May 29, 2000)

The Patent Term Extension is 0 day(s). Any patent to issue from the above-identified application will include an indication of the 0 day extension on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Extension is the filing date of the most recent CPA.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

John L. Schenk, Fort Collins, CO;

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CHANGE OF CORRESPONDENCE ADDRESS Patent Address to: Mail Stop Post Issue Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Patent Number	7208265
	Issue Date	2007-04-24
	Application Number	09/478299
	Filing Date	2000-01-05
	First Named Inventor	John L. Schenk
	Attorney Docket Number	22091-701

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I am the:

☐ Patentee.

☐ Assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

☒ Attorney or agent of record. Registration Number 48665.

Signature / Nicole A. Ressue /

Typed or Printed Name Nicole A. Ressue

Date 2009-04-20	Telephone 970-224-3100

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☒ *Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Post Issue, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Electronic Acknowledgement Receipt	
EFS ID:	5180433
Application Number:	09478299
International Application Number:	
Confirmation Number:	1509
Title of Invention:	METHOD OF CRYOPRESERVING SELECTED SPERM CELLS
First Named Inventor/Applicant Name:	John L. Schenk
Correspondence Address:	Craig Miles Santangelo Law Offices P C 125 South Howes Street 3rd Floor Fort Collins CO 80521 US 650-849-4910 -
Filer:	Nicole Anderson Ressue/Cheryl A. Swanson
Filer Authorized By:	Nicole Anderson Ressue
Attorney Docket Number:	22091-701
Receipt Date:	20-APR-2009
Filing Date:	05-JAN-2000
Time Stamp:	12:52:39
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Change of Address	XY-FrozenUS-09478299-pat-PTO.pdf	52056	no	1
			079504fd3235f75e120a299849a234ae28113826		
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Total Files Size (in bytes):			52056		
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					



EXPRESS MAIL LABEL No.: EM316123495US

UNITED STATES PATENT AND TRADEMARK OFFICE

Title: Method of Cryopreserving Selected Sperm Cells
Inventors: John L. Schenk
United States Patent No.: 7,208,265
Application Number: 09/478,299
Filing Date: January 5, 2002

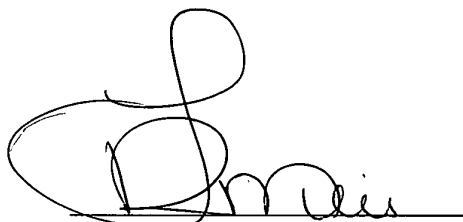
CERTIFICATE OF EXPRESS MAILING

I, Justin Ruth Miles, hereby certify to the truth of the following items:

1. I am an employee of CR MILES P.C., 405 Mason Court, Suite 119, Fort Collins, CO 80524.
2. I have this day forwarded the attached Statement under 37 CFR 3.73 (b) (1 page(s)) with the United States Postal Service as "Express Mail" for mailing to:

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dated this 29 day of January, 2010



Justin Ruth Miles



2-1-10

Zhu

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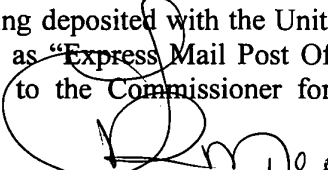
Title: Method of Cryopreserving Selected Sperm Cells
Inventors: John L. Schenk
United States Patent No.: 7,208,265
Application Number: 09/478,299
Filing Date: January 5, 2002

Commissioner for Patents
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CERTIFICATE UNDER 37 C.F.R. § 1.10 FOR "EXPRESS MAIL"

Date of Deposit: January 20, 2010 Express Mail Label No.: EM316123495US

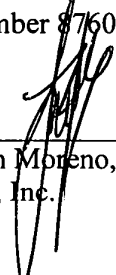
I hereby certify that this document is being deposited with the United States Postal Service on the date indicated above in an envelope as "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 and addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.


Justin Ruth Miles

**REVOCATION OF POWER OF ATTORNEY WITH NEW POWER OF ATTORNEY
AND CHANGE OF CORRESPONDENCE ADDRESS**

I, Juan Moreno, CEO of XY, Inc. being the assignee of record under §37 C.F.R. 3.71 of the entire interest in the above-identified application, hereby revoke all previous powers of attorney and appoint XY, Inc., having an office at 22575 State Highway 6 South, Navasota, TX 77868 USA having Customer Number 87602 including Cindee Ewell whose registration number before the United States Patent and Trademark Office is 59,363 and Ryan Christensen whose registration number before the United States Patent and Trademark Office is 60,291 as my attorneys to prosecute this application and to transact all business in the United States Patent and Trademark Office therewith. Please change the correspondence address for the above-identified application to the address associated with Customer Number 87602.

Dated this 25 day of January, 2010

By: 
Juan Moreno, CEO
XY, Inc.



PTO/SB/98 (07-08)

Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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STATEMENT UNDER 37 CFR 3.73(b)Applicant/Patent Owner: John L. SchenkApplication No./Patent No.: 7,208,265Filed/Issue Date: April 24, 2007

Titled:

Method of Cryopreserving Selected Sperm Cells

XY, Inc.

, a corporation

(Name of Assignee)

(Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. ☒ the assignee of the entire right, title, and interest in;
2. ☐ an assignee of less than the entire right, title, and interest in
(The extent (by percentage) of its ownership interest is _____ %); or
3. ☐ the assignee of an undivided interest in the entirety of (a complete assignment from one of the joint inventors was made)

the patent application/patent identified above, by virtue of either:

- A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 010503, Frame 0068, or for which a copy therefore is attached.

OR

- B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____

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3. From: _____ To: _____

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☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

- ☐ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Cindee R. Ewell

Signature

1-25-10

Date

Cindee R. Ewell

Printed or Typed Name

Attorney of Record

Title

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
09/478,299	01/05/2000	John L. Schenk	22091-701

87602
Inguran LLC
22575 State Highway 6 South
Navasota, TX 77868

CONFIRMATION NO. 1509
POA ACCEPTANCE LETTER



Date Mailed: 03/09/2010

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 01/29/2010.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

/atesfai/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101



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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
09/478,299	01/05/2000	John L. Schenk	22091-701

Craig Miles
Santangelo Law Offices P C
125 South Howes Street
3rd Floor
Fort Collins, CO 80521

CONFIRMATION NO. 1509
POWER OF ATTORNEY NOTICE



Date Mailed: 03/09/2010

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 01/29/2010.

- The Power of Attorney to you in this application has been revoked by the assignee who has intervened as provided by 37 CFR 3.71. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

/atesfai/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101



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APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	FILE WRAPPER LOCATION
09/478,299	7208265	1655	9200



Correspondence Address/Fee Address Change

The following fields have been set to Customer Number 114661 on 05/13/2013

- Correspondence Address
- Maintenance Fee Address

The address of record for Customer Number 114661 is:

114661
XY, LLC
22575 State Highway 6 South
Navasota, TX 77868